Environmental and anthropogenic factors affecting the respiratory toxicity of volcanic ash

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Abstract

The potential adverse health outcomes of exposure to inhalable volcanic ash have been a long-standing concern, especially as it is known that respirable-sized particles can potentially contribute towards the onset or exacerbation of respiratory and cardiovascular diseases. In recent years, substantial knowledge of the posed respiratory hazard, alongside extensive characterisation of the physicochemical properties of volcanic ash that may influence its biological reactivity, has been obtained. However, knowledge of how external factors, including the volcanic plume, itself, and anthropogenic pollutants, may alter potential ash toxicity and contribute to any adverse respiratory health effects is limited.

Using novel, multidisciplinary approaches and methods, across geochemistry and particle toxicology, this thesis is the first to assess whether ash particle coatings, which originate from in-plume reactions with volcanic gases, can contribute to or alter ash toxicity, as well as if concomitant exposure to volcanic and anthropogenic pollutants poses a greater respiratory hazard than the individual respiratory toxicities of either anthropogenic pollution or volcanic ash alone.

Combined exposure to respirable-sized volcanic and diesel exhaust particles was shown to induce (pro-)inflammatory response in a multicellular human lung model *in vitro*, implying a potentially-greater hazard of simultaneously inhaling both particle types. However, no significant toxicological effects of in-plume processing or co-exposures with complete (gasoline) exhaust were found. The fact that sulphate salts dissolve rapidly, likely prior to cellular uptake, is a finding which helps explain why the salt-laden samples had no toxicological impact.

Although further work is required to derive a more comprehensive understanding of the interactions of volcanic ash and urban pollutants in the ambient air and potential impacts of co-exposures, the findings of this thesis provide the first evidence which can be used towards the assessment of respiratory health hazard following the onset of new volcanic activity where exposed communities live in heavily polluted urban areas.

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Declaration

I declare that this thesis, submitted for the degree of Doctor of Philosophy at Durham University, is my own work and not substantially the same as any which has been previously submitted at this or any other university. Where appropriate, I have clearly indicated the contributions of colleagues to fully acknowledge all collaborative work.

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The Beginning.

Dedication

Za mamu, tatu, seku, Gitu i Luku.

“The care, the love, the memories

We are the story of one”

T. Holopainen (Nightwish)

Part I

Overview

Chapter 1

Introduction

1.1 Thesis rationale

Volcanic ash is a product of volcanic eruptions, generated by fragmentation of magma and volcanic rocks, consisting of fine fragments of magmatic glass, crystals, and other rocks (lithic material) with a diameter below 2 mm (White and Houghton, 2006). During its eruption, transport and deposition, ash poses a range of hazards to human and animal health, infrastructure, air traffic and agriculture over widely-varying spatial and temporal scales (Dingwell et al., 2012). With nearly 10 % of the world's population living near a historically active volcano (Small and Naumann, 2001), acute and chronic human exposure to inhalable volcanic ash particles has been a long-standing health concern (Horwell and Baxter, 2006), especially as it is known that respirable-sized particles can potentially contribute towards adverse health effects, such as the onset or exacerbation of respiratory and cardiovascular diseases (WHO, 2013). Recently, ambient particulate matter has been classified as a lung carcinogen (Loomis et al., 2013), thus, reinforcing the need to understand the potential adverse outcomes of exposure to inhalable ash.

Substantial knowledge of the posed respiratory hazard, alongside extensive characterisation of the physicochemical properties of volcanic ash that may influence its biological reactivity (*e.g.*, size, shape, surface area, mineralogical composition and physicochemical properties of ash surfaces), has been obtained in recent years (*e.g.*, Damby et al., 2017, Hillman et al., 2012, Horwell et al., 2010, Horwell, 2007), leading to a better understanding of the structure-toxicity relationship (Damby et al., 2016, Horwell et al., 2012). The crystalline silica content of ash and its properties have been studied in detail and characterised for different volcanic settings (*e.g.*, Damby et al., 2018, Horwell et al., 2014, Damby et al., 2013, Horwell et al., 2012), due to its known carcinogenicity (IARC, 1997) and risk of developing silicosis (an untreatable lung disease specific to silica inhalation) in industrial settings. The iron-induced generation of reactive oxygen species (*e.g.*, free radicals) from ash particle surfaces when in contact with biological fluids has been identified as another mechanism by which ash may pose a respiratory hazard (Horwell et al., 2017, Horwell et al., 2007). However, knowledge is limited of how

external factors, including the volcanic plume, itself, and anthropogenic pollutants, may alter potential ash toxicity and contribute to any adverse respiratory health effects.

The objectives of this thesis were twofold:

Objective 1: to determine how interactions of ash particles with the gaseous volcanic plume environment may alter their toxicity.

Objective 2: to determine the respiratory hazard of exposures to volcanic particles concomitantly with anthropogenic pollution.

To address *Objective 1*: During a volcanic eruption, fine-grained ash is generated primarily through magma fragmentation and is buoyantly lofted into an eruption cloud, the plume from which can be transported over great distances. During transport, the ash particles interact with gases and aerosols within the volcanic plume, as well as with trace atmospheric gases (Usher et al., 2003), potentially changing their surface composition and reactivity which may impact their toxic potential (Horwell and Baxter, 2006). Hence, the understanding of volcanic gas-ash interactions is highly relevant for health hazard assessment of volcanic ash. In the existing literature, it has been postulated that the presence of adsorbed species (*e.g.*, sulphate and halide salts) on ash surfaces may potentially affect the respiratory system through leaching in contact with lung fluid (*e.g.*, Witham et al., 2005), but this has not been adequately addressed in studies to date. This study was the first to assess whether the precipitation of salts on the surface of volcanic ash, from interactions of ash with the gaseous volcanic plume environment, might impact ash respiratory toxicity *in vitro*.

Regarding *Objective 2*, as ash particles fall from the plume, they may also interact with anthropogenic pollution, such as vehicle exhaust particles and gases, which can be substantial in urban settings. Communities resident in urban areas located near active volcanoes are thus commonly exposed to ash concomitantly with urban air pollutants. A prime example of this is Mexico City, which was named by the United Nations as the world's most polluted city in 1992 (WHO, 1992) and

sits just 70 km from the frequently-erupting Popocatépetl volcano. There is a clear knowledge gap in the potential health hazard and impacts from concomitant exposure to volcanic and anthropogenic emissions, especially whether this may pose a greater respiratory hazard than inhaling respirable volcanic ash and vehicle emissions separately. Therefore, this thesis presents the first study to evaluate the hazard of simultaneously inhaling anthropogenic pollutants and volcanic ash. This was done through *in vitro* toxicological appraisal of the reactivity of volcanic ash with diesel exhaust particles and complete gasoline exhaust.

The studies in this thesis used novel, multidisciplinary approaches and methods, across geochemistry and particle toxicology, in order to build an evidence base where currently there is limited knowledge. The findings of this thesis will be useful to governmental agencies and hazard management organizations, worldwide, so that they can make informed decisions on whether to advise that citizens take action to protect themselves during periods of intense exposure to both urban pollution and volcanic ash.

1.2 Thesis Background

Objective 1 was based on studies which have recognised the role of volcanic ash in the scavenging of volatiles in volcanic plumes and, thus, in dispersal of adsorbed materials into the environment where, upon their release, they can have negative impacts on, *e.g.*, vegetation, animals and people (*e.g.*, Ayris and Delmelle, 2012, Witham et al., 2005). Investigation of potential changes in ash toxicity resulting from the presence of soluble surface coatings was mainly inspired by ash leachates studies which hypothesise that freshly erupted ash particle coatings, if leached from the surface when in contact with lung fluid, could be potentially responsible, at least in part, for the reported short-term human health effects following eruptions, including irritation of the respiratory tract (Horwell and Baxter, 2006, Witham et al., 2005).

Objective 2 stemmed from the British Geological Survey's report to the UK Government on characteristics of a future large, effusive Icelandic eruption, which highlighted the urgent need to evaluate the role of mixing volcanic emissions with anthropogenic pollutants and whether this would affect the individual respiratory toxicities of either anthropogenic pollution or volcanic ash (Loughlin et al., 2012). Although some investigations, to date, have indicated that ash is less toxic than particles in the ambient air, the UK Health Protection Agency (now Public Health England) identified comparative toxicology of ambient air particles and volcanic ash as a key area of uncertainty and made recommendations to prioritise research within this area (Kar-Purkayastha et al., 2012).

1.3 Hypotheses

The current study had multiple hypotheses to address the two main objectives. These are described, below, along with research aims for addressing each hypothesis, as follows:

Hypothesis 1: Volcanic ash toxicity is altered by the precipitation of salts on the surface of volcanic ash, from interactions of ash with the gaseous volcanic plume environment.

- To produce simulated volcanic ash material using different substrates.
- To produce a salt loading on the particle surfaces using a simulated plume environment.
- To assess the potential toxicity to the lungs of exposure to (simulated) ash that has interacted with gases.
- To determine if surface interactions and coatings control *in vitro* toxicity compared to pristine ash.
- To determine the bio-solubility of ash surface coatings following lung deposition, by measuring dissolution of species adsorbed onto the particle surface using water and simulated lung fluid.

Hypothesis 2: Combined exposure to volcanic ash and urban pollution poses a greater respiratory hazard than exposure to either, independently.

- To determine and compare individual respiratory toxicities of volcanic ash and a particulate urban pollutant (*i.e.*, diesel exhaust particles) *in vitro*.
- To replicate and determine the effects of combined exposure to urban pollutants and volcanic ash in the lungs using *in vitro* experiments by assessing if the presence of ash affects the measured toxicity of diesel exhaust particles and complete gasoline exhaust.
- To evaluate whether any enhanced toxicity following combined exposures is additive or synergistic in its effect.

Hypothesis 3: The toxic effects of combined exposures will vary with volcanic ash composition.

- To determine the toxicity of different types of volcanic ash in combined exposures, and individually, to evaluate whether the ash (magmatic) composition influences the outcome of the combined exposures in *in vitro* experiments.

Hypothesis 4: Interaction of volcanic ash with vehicle exhaust will result in gas uptake by particle surfaces, potentially creating compounds which are not usually present on the ash surface.

- To evaluate the capacity of ash to uptake urban pollution gases.
- To investigate the mode of potential interactions and the reaction products.

1.4 Approach

In vitro experiments throughout this thesis were performed using a sophisticated 3D multicellular human lung model mimicking the alveolar epithelial tissue barrier (Rothen-Rutishauser et al., 2005), cultured at the air-liquid interface. This was the first time that volcanic ash has been exposed to a multicellular model and was also

the first time that volcanic ash has been applied in its pristine, dry state to cell surfaces. Further, a characterisation method of ash with regards to lung fluid-soluble elements has been set up and performed for the first time at the Department of Earth Sciences, Durham University. This was done concurrently with an international round-robin exercise for validation of a laboratory protocol for the assessment of hazards from leachable elements in volcanic ashfall using different solvents. Technique development has, therefore, been an important aspect of this work and is discussed, in detail, in ***Chapter 3***.

1.5 Thesis Outline

Part I of this thesis gives an overview of the research topic and methodologies. ***Chapter 2*** presents the background to contextualise the studies reported in this thesis. It gives an overview of the fate of inhaled particles within the respiratory system, discusses the health-pertinent physicochemical characteristics of particles, mechanisms of particle-induced toxicity and associated pulmonary diseases, summarises current understanding of the respiratory hazard of volcanic ash and volcanic gas-ash interactions within an eruption plume and in the urban environment, and finally, presents an overview of exposure to urban pollutants (*e.g.*, vehicle exhaust emissions), and related hazards. ***Chapter 3*** provides a detailed description of the main analytical and experimental techniques (repeatedly) used across the studies in this thesis, including isolation of the respirable ash fraction, physicochemical characterisation of ash, description of the 3D *in vitro* multicellular model of the lungs and biochemical analyses used to assess cellular response to particle treatments. Where necessary, the research chapters refer back to the methods outlined in this chapter.

Part II (***Chapter 4***) presents the experiments to assess the effects of salt-laden ash particles (from interactions of ash with the gaseous volcanic plume environment) on the *in vitro* multicellular lung model and addresses *Hypothesis 1*, above. Particles were produced through simulated high-temperature in-plume processing using analogue substrates, *i.e.*, pulverised synthetic volcanic glass and

natural pumice. The samples were characterised with regards to water-soluble and lung fluid-soluble elements that may relate to bio-reactivity of ash.

Part III addresses the anthropogenic factors (*i.e.*, from urban pollutants) which may potentially alter ash toxicity. ***Chapter 5*** reports the results of a first investigation into the biological impact of concomitant exposure to respirable volcanic ash and a model urban pollutant, *i.e.*, standardised diesel exhaust particles and addresses *Hypothesis 2*, above. This landmark work is published in Particle & Fibre Toxicology journal. ***Chapter 6*** presents findings from the assessment of the biological impact of the combined exposure to respirable volcanic ash and freshly-generated complete gasoline exhaust, thus addressing *Hypotheses 2* and *3*. This investigation follows and builds on the experiments discussed in ***Chapter 5*** in order to consider the additional, gaseous component of an exhaust in contrast to using only the particulate fraction, to assess the effects of ash exposure in an urban environment. This study is published in Environmental Pollution journal. ***Chapter 7*** discusses the attempt to address *Hypothesis 4* and investigates whether vehicle exhaust gases interact with the surface of volcanic ash in order to evaluate if ash falling through urban atmospheres interacts with the existing airborne urban pollution, hence providing a mechanism for interactions in the lung between cells and the pollutants.

Finally, ***Part IV*** concludes the thesis. Here, ***Chapter 8*** provides an overview of the key findings of this research and discusses the contributions made to the existing understanding of the respiratory hazard of volcanic ash. It also addresses the wider implications relevant for hazard assessment and presents an outlook in relation to the future research perspectives in this area.

The ***Appendices*** include supporting data and publications that have resulted from this research.

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Chapter 2

Background

2.1 Introduction

The focus of this thesis is the respiratory hazard of volcanic ash and external factors, specifically interaction with volcanic gases and urban pollution, which may affect the potential adverse effects observed in the lung, post-exposure.

This chapter contains a background to the topic, starting with a general overview of the fate of inhaled particles within the respiratory system and the particle interactions with lung components. Physicochemical characteristics of particles and mechanisms of particle-induced toxicity are then discussed, in-depth, and specifically noted for volcanic ash. The common diseases associated with particle exposures are then briefly presented. This is followed by a review of current knowledge on potential respiratory hazard following exposures to volcanic ash. Relevant volcanic gas-ash interactions within an eruption plume are discussed. Finally, an overview of exposure to urban pollutants (*e.g.*, vehicle exhaust emissions), and related hazards, are presented.

2.2 Inhalation of particles

In the process of breathing, air from the environment is introduced into the respiratory system where it travels through the respiratory tract, with the ultimate goal to reach the air-blood barrier, where gas exchange occurs, in order to transfer oxygen to the body. In this process, numerous particles (of anthropogenic and natural/geogenic origin), microorganisms and potentially harmful contaminants from the atmosphere are introduced in the airways (Phalen et al., 1995, Nicod, 2005). Subsequent particle-lung interactions (*Section 2.3*) may lead to the development of pulmonary diseases (Gehr et al., 2000) (*Section 2.4*).

Structural barriers in the lung somewhat protect the respiratory system from harmful foreign substances and particles (Nicod, 2005). The structures of the respiratory tract are grouped in three main regions (*e.g.*, ISO, 1983, Oberdorster et al., 2005). The extra-thoracic region includes the airways of the head and neck down through the larynx (*Fig. 2-1*). The tracheobronchial region includes the

trachea, ciliated bronchial airways (*i.e.*, bronchial tree) and the terminal bronchioles. The alveolar region (gas-exchange region) comprises of respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli. Being the most relevant structure to the focus of this thesis, alveoli are further described in the following section.

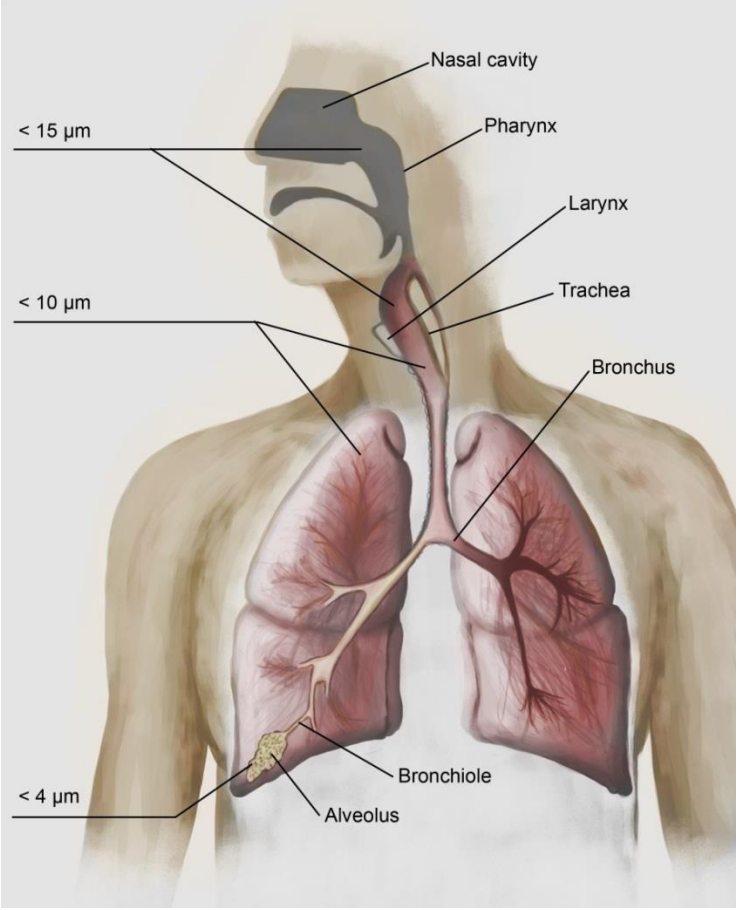

Figure 2-1: Gross anatomy of the respiratory system and particle deposition sites as a function of particle size (*adapted from: Horwell and Baxter (2006), produced by P-Y. Tournigand*).

2.2.1 Alveoli

Alveoli are comprised of the air-blood barrier where the transfer of gases between the air and blood takes place over the available surface area of approximately 140 m^2 (Gehr et al., 1978), which makes up the majority of the vast internal surface area of the lungs. The air-blood barrier is made of three parts (*Fig. 2-2*): the alveolar epithelium, the interstitium and the capillary endothelium.

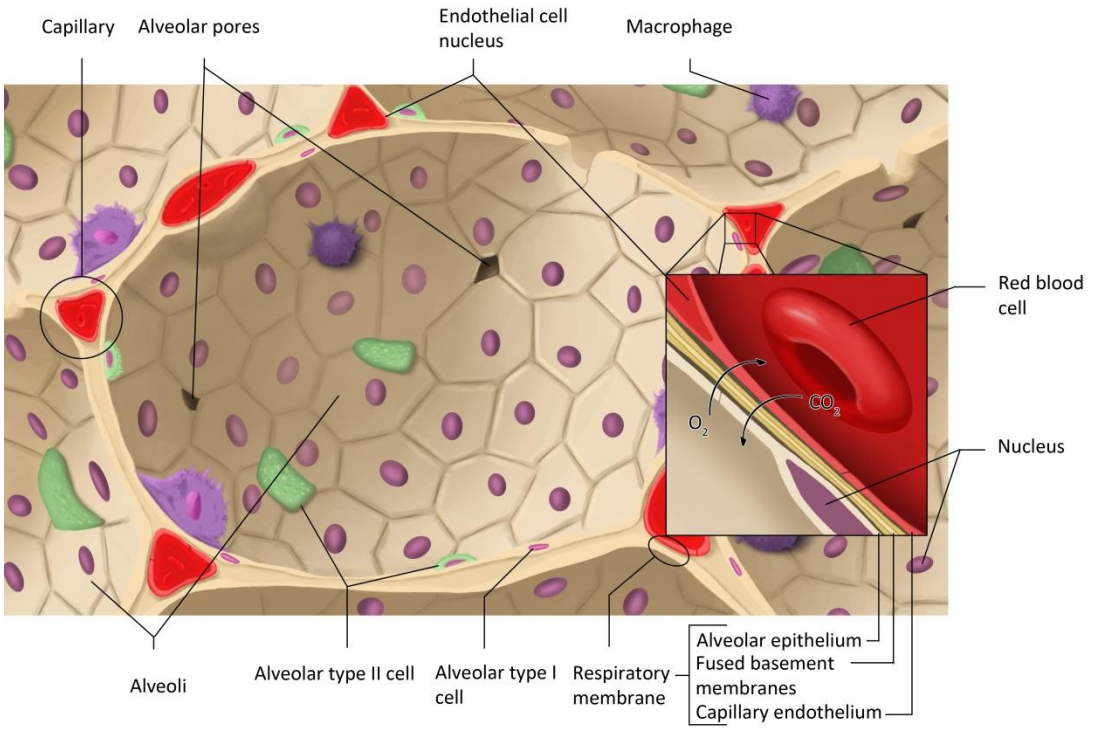

Figure 2-2: Respiratory membrane – air-blood barrier (*adapted from: Mescher (2013) (http//:www.accessmedicine.com), produced by P-Y. Tournigand*).

The alveolar epithelium is made up of two types of alveolar epithelial cells, type I and type II (Dobbs and Johnson, 2007, Crapo et al., 1983) (*Fig. 2-2*). Type I cells form the basic structure of alveoli, covering about 95% of the alveolar surface (Crapo et al., 1982, Williams, 2003). Type II cells produce and secrete pulmonary surfactants (*see below*) and they have an important role in the regeneration of the alveolar epithelium, being able to differentiate into both type I and type II cells (Fehrenbach, 2001, Mason and Williams, 1977). They play a key role in particle-induced inflammation, contributing to lung immune responses by secreting a variety of (pro-)inflammatory mediators, including cytokines and chemokines - a group of proteins involved in cell signalling (Dobbs et al., 2010, Fehrenbach, 2001). It is the type II-like epithelial cell line (A549) that composes a layer in the multicellular lung model (described in ***Chapter 3***) used for toxicology experiments in this thesis.

The surface of the alveolar epithelium, like the airway surfaces, is covered by a thin (0.1 - 0.5 µm) liquid layer, *i.e.*, the lining fluid, which is mainly comprised of

pulmonary surfactant (Notter, 2000), a complex mixture of lipids (mainly phospholipids) and proteins produced by the epithelial cells (Hawgood, 1997). The role of the surfactant is to protect and stabilise the alveoli by reducing surface tension at the air-liquid interface (Gehr et al., 2000, Wright, 2005). Upon deposition, particles interact with surfactant film and are wetted and may also be coated with surfactant components, before they come into contact with the epithelium and alveolar macrophages (Gehr et al., 1990, Gehr et al., 1996, Schürch et al., 1990). Interactions between particles, surfactant and alveolar epithelium are further discussed in the following *Section 2.3*.

2.3 Particle-lung interactions

The fate of inhaled particles depends on various factors, including their site of deposition (*Section 2.3.1*), the structures particles interact with (*Section 2.3.2*), including different cell types and the surfactant layer at the air-liquid interface, and particle physicochemical properties (*Section 2.3.3*). Hence, these factors are important as they may affect the toxic potential of particles.

2.3.1 Deposition of inhaled particles

The location of particle deposition in the respiratory tract is dictated by particle properties (*e.g.*, size, shape; see *Section 2.3.3*), but also by lung anatomy and breathing pattern of the host (Gehr and Heyder, 2000). It is predominantly affected by three different mechanisms of particle motion in the airways: sedimentation, impaction and diffusional motion of particles (Schulz et al., 2000, Möller et al., 2010), which lead to heterogeneous particle deposition in the respiratory tract (see Gehr and Heyder, 2000).

Larger particles (inhalable fraction; see *Section 2.3.3.1*) are mainly deposited in the extra-thoracic region, which includes the nose, mouth, pharynx, and larynx, due to impaction as they are unable to follow the streamlines of the airflow at bifurcations where the airflow deviates. Hence, such particles are filtered out of the airstream and cannot penetrate down to the deep lung. Particles > 0.5 µm can pass

through the large airways and will be deposited in the lung periphery due to sedimentation, when the airflow no longer supports a particle. Particles < 0.5 µm can behave like diffusing gas molecules and will be dispersed due to the Brownian motion of the air molecules, which enhances the chance of their subsequent deposition in all regions of the airway (Schulz et al., 2000, Möller et al., 2010).

2.3.2 Particle clearance, retention and translocation

Particles are eliminated from the respiratory tract by long- and short-term clearance mechanisms, depending on the particle deposition site (Kreyling et al., 2007). Particles deposited in the airways of the extra-thoracic and tracheobronchial regions are mainly cleared by mucociliary transport. This refers to particles being transported in mucus, which covers the airways, by beating cilia out of the airways and towards the larynx from where it is swallowed into the stomach, expectorated, or blown from the nose. The removal of particles *via* this mechanism is fast and the majority of particles are cleared out within about 24 hours (Morgan et al., 2004). However, smaller particles may penetrate through the mucus into the epithelium where they are retained until cleared by other mechanisms, such as uptake (*see below*) by airway phagocytic or epithelial cells (Geiser et al., 2005).

When deposited in the non-ciliated alveolar region, particle clearance kinetics is slower. Some particles may be transported mechanically along the epithelium towards the ciliated airway epithelium where they are cleared by mucociliary transport (*as described above*), or through the epithelium into the interstitial spaces or subsequently enter circulation (including lymphatic system), where they are transported to other organs of the body (Kreyling et al., 2007). Similarly, engulfed particles may be transported within macrophages out of the alveolar region. The mechanism of particle uptake into the 'specialised', phagocytic cells such as macrophages and dendritic cells, whose main function is to remove foreign material from the body, is known as phagocytosis. Once internalised, acidic conditions within compartments of the macrophage interact with particles to dissolve them. However, some particles are not efficiently cleared out and can remain in the lung

for a longer time, hence, they can potentially trigger an immune response and/or mediate the onset of diseases (Donaldson and Borm, 2006).

2.3.3 Physicochemical properties of inhaled particles

Physicochemical properties of particles control their toxicity and dosimetry parameters, such as deposition, retention, dissolution, clearance and translocation, in the lungs. Hence, particle properties are important to be characterised and considered in order to deduce their contribution to, and mechanisms of, toxicity (see *Section 2.3.4*). Those most relevant for potential adverse respiratory health effects are discussed in the following sections.

2.3.3.1 Particle size, shape and specific surface area

Particle size relates to the potential for particles to cause respiratory health effects, as it dictates the particle deposition site within the respiratory tract (*Fig. 2-1*, see *Section 2.3.1*). Conventional health-related size fractions are defined based on aerodynamic particle diameter (ISO, 1995, Harrison et al., 1996). The aerodynamic diameter is a means of standardising individual particles as unit-density spheres with the same settling velocity as irregularly shaped particles (Solomon and Costa, 2010). It is commonly used to describe ambient particulate matter (PM). The 'Inhalable' fraction is defined as particles which enter the body during breathing through the nose and/or mouth (< 100 µm). Inhaled particles which can penetrate the respiratory system beyond the larynx (< 10 µm) are considered as the 'thoracic' fraction. 'Respirable' particles (< 4 µm) can penetrate to, and deposit in, the alveolar region of the lung. It has been shown that smaller particles are more potent than larger particles at inducing adverse effects, such as inflammation (Brown et al., 2001), especially in the case of air pollution and its ultrafine fraction (< 100 nm) (Hester et al., 1998). Further, there is evidence that a larger particle which is non-toxic, such as titanium dioxide, may be toxic if in the ultrafine size range (Ferin et al., 1990).

Similarly to size, particle shape (*e.g.*, spherical, non-spherical, sheet-like, fibres) has an impact on the deposition site within the respiratory tract, as it can

affect the aerodynamic behaviour of the particles. For example, fibres may penetrate deep into the lung due to the alignment of fibres along the airflow where, during extended periods of residence, they can incite cancerous transformations (Warheit et al., 1984).

Post-deposition, both particle size and shape can affect phagocytosis (engulfing of particles by macrophage, discussed in *Section 2.3.2*). For example, a study by Clift et al. (2008) showed that the uptake of particles of 200 nm diameter is slower than for smaller sized particles (20 nm). Further, it has been shown that macrophages (size ranging from 10 to 15 µm diameter) are not able to completely engulf and remove long (tens of µm length) fibre particles from the alveolar epithelium (Champion and Mitragotri, 2006), resulting in frustrated phagocytosis, which leads to cell death (Schinwald and Donaldson, 2012). Particle size and shape also influence toxicity by dictating the surface area of a particle.

Respirable volcanic ash (< 4 µm) is mainly generated in explosive eruptions (*e.g.*, Chaitén volcano, Chile), and especially in phreatomagmatic eruptions (*i.e.*, where there is water-magma interaction), and during collapses of viscous lava domes (*e.g.,* Soufrière Hills volcano, Montserrat) (Horwell, 2007, Horwell et al., 2010a, Zimanowski et al., 2003, Rose and Durant, 2009). In this study, two samples of volcanic ash are used, from Soufrière Hills and Chaitén volcanoes (see ***Chapter 3***, *Section 3.2*), both of which comprised 12 vol. % sub-4 µm material, hence indicating that these ash deposits may pose a potential health concern.

With regards to the shape of respirable ash particles, the morphology generally observed, and that of samples used in this study, is mostly blocky and angular (*e.g.*, Damby et al., 2016, Lahde et al., 2013, Horwell et al., 2013, Hillman et al., 2012, Le Blond et al., 2010, Horwell and Baxter, 2006). However, in some cases, the occurrence of occasional fibre-like particles has been reported (Horwell et al., 2010a, Reich et al., 2009).

The specific surface area (SSA) of particles, defined as the surface area per mass of a particle, is another important physical property which may influence

particle reactivity and its toxic potential. Larger surface area, being inversely related to particle size, is associated with smaller particles but also with the increased toxic potential of particles (Duffin et al., 2007, Duffin et al., 2002). This is likely related to the fact that more surface is available for interactions with the components of the lung structures.

Reported values for volcanic ash from different eruptions typically range from 1 to 3 m^2/g (*e.g.*, Delmelle et al., 2005, Horwell et al., 2013, Horwell et al., 2007, Horwell et al., 2010b, Le Blond et al., 2010) as measured by commonly-used gas adsorption techniques (see ***Chapter 3***). Exceptionally, higher surface area has been measured in ash from phreatomagmatic eruptions (> 4 m^2/g; see Gislason et al. (2011)) or ash altered by weathering or hydrothermal alterations, with SSA exceeding 6 m^2/g (Horwell et al., 2003a, Delmelle et al., 2005).

2.3.3.2 Particle composition

Particle composition varies not only among different types of particles but also within individual particles, with regards to both the surface and the core of a particle. It is necessary to distinguish the potential differences since, upon contact with lung structures, the surface of an insoluble particle will undergo biochemical interactions and, hence, chemical and physical change (Kendall et al., 2004, Möller et al., 2010). For example, water-soluble or lipid-soluble particle surface compounds will be dissolved in the lining fluid and potentially transferred to the blood, while the insoluble residue of the particle may remain in the lungs for an extended period of time until removed by a clearance mechanism (see *Section 2.3.2*).

The bulk composition of volcanic ash can vary depending on the chemical composition of the source magma which is composed mainly of the major crustal elements silicon (Si), aluminium (Al), iron (Fe), magnesium (Mg), calcium (Ca), sodium (Na) and potassium (K) (Heiken and Wohletz, 1985). Actually, magma composition indirectly influences other physical properties of ash, in the way it dictates eruption type (*i.e.*, explosivity) via its viscosity. For example, silica and crystal-rich magmas have higher viscosity and tend to erupt explosively, hence

generating finer ash with correspondingly greater specific surface area (Lesher and Spera, 2015, Mysen and Richet, 2005). Using the bulk composition of igneous rock, or ash, there is a nomenclature which typically describes magma type using a total alkali (Na_2O + K_2O) versus silica (SiO_2) plot (Le Maitre et al., 2002, Fisher and Schmincke, 1984), where low SiO_2 and alkali content is defined as 'basaltic', intermediate compositions are defined as 'andesitic' and 'dacitic' and high SiO_2 is 'rhyolitic'. The two ash samples used in this study represent different magmatic compositions (andesitic and rhyolitic, as described in ***Chapter 3***, *Section 3.2*). The presence and the role of crystalline silica in volcanic ash toxicity are discussed in *Section 2.5*.

As for the chemical composition of volcanic ash surfaces, they may comprise the same constituents as the bulk, if the particle is homogenous, but will also likely include additional elements sourced from magmatic volatile species in the volcanic plume including sulphur (S), chlorine (Cl) and fluorine (F) (Wohletz and Heiken, 1992). However, some elements such as Si, Mg, S, Cl and F have been found to be enriched in the surface compared to the bulk while some such as Fe, Ca and Na and K have been found be depleted in the surface (Delmelle et al., 2007). This discrepancy is believed to arise as a result of various physical (*e.g.*, grinding), but mainly chemical processes (*e.g.*, oxidation and/or acid leaching and subsequent dissolution of particle bulk) that ash undergoes whilst interacting with gases and condensates in the eruption plume and with the atmosphere during plume transport and dispersion (Delmelle et al., 2007, Rose, 1977, Oskarsson, 1980, Taylor and Stoiber, 1973). Such processes, in general, modify the chemical composition, and likely the reactivity, of the ash surface, potentially affecting its toxic potential.

These in-plume processes are believed to be responsible for the presence of soluble compounds found on pristine ash surfaces, such as sulphates ($CaSO_4$, Na_2SO_4) and halides, including NaCl, NaF, $CaCl_2$, CaF_2, K_2SiF_6, Na_2SiF_6 and AlF_3 (Cronin et al., 2003, de Moor et al., 2005, Delmelle et al., 2007, Gislason et al., 2011). The mechanisms of their emplacement onto the ash surface are discussed in detail in *Section 2.6*.

2.3.4 Mechanisms underlying adverse effects of inhaled particles

The experimental work of this thesis was designed to investigate the ability of particles to cause oxidative stress, cell death (cytotoxicity) and (pro-)inflammatory response in the multicellular alveolar airway barrier *in vitro* model (***Chapter 3***). Hence, these common toxicological processes and mechanisms relevant for any inhaled particle-induced responses are briefly outlined below.

2.3.4.1 Oxidative stress

Oxidative stress is a state of imbalance between antioxidants and oxidants which occurs either due to excessive production of oxidants or depletion of antioxidants (MacNee and Rahman, 2001). Antioxidants (*e.g.*, glutathione) protect the cells from the potential damaging effects of oxidants, such as free radicals and reactive oxygen species (ROS) which can be introduced into the body by inhaled particles.

ROS are oxygen-containing chemical species that possess an unpaired electron, making them highly reactive and potentially damaging to cells (Kelly, 2003, Fubini and Arean, 1999, Fubini et al., 1995). They can be released by phagocytic cells upon ingestion of inhaled (especially fibre-like) particles as part of their activity or can be generated directly at the particle surface (MacNee and Rahman, 2001, Donaldson and Borm, 2006). The particle-generated ROS are considered a key mechanism of particle-induced oxidative stress, as indicated in numerous *in vivo* and *in vitro* studies (see *e.g.*, Stone and Donaldson, 2010). This is also believed to be a likely mechanism involved in volcanic ash toxicity (Horwell et al., 2017, Horwell et al., 2007, Horwell et al., 2003a), potentially, but not exclusively, resulting from the presence of iron on the ash surfaces. Particle-induced oxidative stress may cause DNA damage and, furthermore, activate other cellular signalling processes (*e.g.*, (pro-)inflammatory signalling), which can initiate inflammation and lead to the development of diseases (*e.g.*, Fubini, 1997, Kane, 1996).

2.3.4.2 Cytotoxicity

Cytotoxicity refers to the ability of inhaled particles to compromise the cell viability, *i.e.*, cause damage to a cell, and eventually cell death, either by inducing apoptosis (programmed cell death) or necrosis (accidental cell death).

2.3.4.3 Inflammation

Inflammation is required for normal health and represents the response of the immune system to a stimulus (*i.e.*, a foreign substance). A healthy response occurs when the cells can counteract the (pro-)inflammatory stimuli and prevent the damage, whereas in case of a continuous stimuli an inflammation which is inappropriate in duration or amplitude may occur, which can further cause a chronic disease (*e.g.*, Lloyd and Marsland, 2017).

Inflammation is considered to be a key response to, and process induced by, respirable particles, including particulate air pollution, asbestos, silica and nanoparticles, as well as their components (such as organics and endotoxins), that further drive lung diseases (*e.g.*, Donaldson et al., 2001, 2000, Schins et al., 2004). A consequence of exposure to such particles is the activation of clearance process (see *Section 2.3.2*) and corresponding interaction of particles with epithelial cells and macrophages, which may result in the upregulation of inflammatory cytokine gene expression in both cell types, and associated cytokine production. Cytokines are a group of proteins with an important role in cell signalling as they, upon their release, affect the behaviour of surrounding cells and, in this way, mediate the immune response. An alteration in such cell signalling pathways, as well as occurrence of cell death and oxidative stress, can lead to decreased particle clearance and hence, may result in inflammation.

Some of the components of ambient particulate matter thought to be responsible for driving such (pro-)inflammatory effects include the ultra-fine and nanoparticle fraction (Donaldson et al., 2000, Brown et al., 2000), and metals (Carter et al., 1997, Gilmour et al., 1996). It has been postulated that particle-induced inflammation, if it persists over a longer period, can either exacerbate pre-

existing disease or even cause new disease in susceptible individuals (Pope III, 2000, Seaton et al., 1995, Donaldson et al., 2001).

2.4 Diseases associated with inhalation of particles

Exposure to particles, in both occupational and environmental settings, has been associated with adverse health effects (acute or chronic) and subsequent exacerbation and/or development of respiratory diseases. The World Health Organization has estimated that 3.7 million persons died prematurely in 2012 due to the effects of ambient air pollution, to a large extent due to respiratory diseases such as chronic obstructive pulmonary disease (COPD), lower respiratory tract infections and lung cancer (WHO, 2016, 2014). Identifying the hazardous factors (*e.g.*, type of particles) enables public authorities to put in place preventive measures and management plans which are important in the reduction of the associated morbidity and mortality. Some of the major chronic lung diseases known or suspected to be caused by inhalation of particles, such as asthma, bronchitis, COPD, pneumoconiosis and cancer, are briefly explained below.

2.4.1 Asthma and bronchitis

Both asthma and bronchitis are inflammatory disorders usually associated with the obstruction of the airway due to allergic responses or local inflammation, which can result in difficulties with breathing (*e.g.*, Bateman et al., 2008). Studies on the effects of urban air pollution have shown that people with such pre-existing conditions, especially elderly people and children, are likely to be more susceptible to particulate exposure (Pope et al., 2004). It was found that even acute exposures of diseased individuals to relatively low levels of respirable particulate matter may increase their symptoms or even result in death (Pope and Dockery, 2006, Dockery and Pope, 1994, Dockery et al., 1993).

In case such inflammatory conditions persist over a longer period of time, leading to further narrowing of the airways (*i.e.*, chronic bronchitis) and breakdown of alveoli surfaces (*i.e.*, emphysema), the disease is characterised as COPD.

2.4.2 Chronic obstructive pulmonary disease

By definition, "*COPD is a common, preventable and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases*" (GOLD, 2017). Airway obstructions are caused by local inflammation and mucus hypersecretion, and are mostly irreversible (Kreyling et al., 2007). The prevalence of the disease is predominantly related to cigarette smoking, however, it has been shown that chronic exposure to high concentrations of air pollution may represent an important risk factor as well (Eisner et al., 2010, Sunyer, 2001).

2.4.3 Pneumoconioses

The pneumoconioses are a suite of fibrotic lung diseases (where the formation of scars occurs in the lung tissues) caused by exposure, predominantly occupational, to high concentrations of airborne particles. There are many documented pneumoconioses induced by different types of dust (see Jones and BeruBe, 2006), but the most studied ones include silicosis, asbestosis, and coal workers' pneumoconiosis caused by silica, asbestos fibres and coal dust, respectively (Morgan and Seaton, 1975). Generally, silicosis and asbestosis are associated with low dose exposures of highly toxic materials, while pneumoconiosis is induced by extremely high exposures to low toxicity materials, over a long period of time. Although, in the case of silicosis, a rapidly progressive form, *i.e.*, acute silicosis, can occur after short periods (< 5 years) of intense exposure (NIOSH, 2002).

Development of silicosis has been a primary concern in volcanic ash exposures, as ash can contain substantial quantities of crystalline silica (in the form of quartz or cristobalite) (see Kar-Purkayastha et al., 2012). However, to date, no cases have been reported (Gudmundsson, 2011).

2.4.4 Lung cancer

Development of lung cancer has been associated with particle exposures, mainly occupational exposures to carcinogenic particles like asbestos and quartz (Morgan

and Seaton, 1975), but also with environmental exposure to air pollution (Cohen and Pope, 1995). Based on the consistent findings across a wide range of studies regarding the carcinogenicity, both outdoor air pollution and particulate matter from outdoor air pollution were recently classified as carcinogenic to humans (Loomis et al., 2013, IARC, 2013).

Generally, there are two mechanisms of particle-induced genotoxicity, namely primary and secondary (Stone and Donaldson, 2010, Borm et al., 2011), likely occurring at the high and low levels of particle exposure, respectively. Primary genotoxicity is characterised by DNA damage in the absence of inflammation, either as a result of direct interaction of particles with DNA (*e.g.*, nanoparticles) or by particle-induced ROS generation forming DNA adducts (a segment of DNA bound to a cancer-causing species (La and Swenberg, 1996)). Secondary genotoxicity is a consequence of particle-induced inflammation, associated with the excessive and persistent formation of ROS from phagocytic cells (Schins, 2002), thus causing a genotoxic insult.

2.5 Respiratory hazard of volcanic ash

Volcanic ash poses a potential respiratory hazard both during and after an eruption, since people will be exposed to ash during ashfall but also, later, due to resuspension by the wind and human activity in dry conditions.

Concerns over the possible adverse respiratory effects of volcanic ash have mostly focused on the crystalline silica content (in the form of quartz and cristobalite), a well-established respiratory hazard (Baxter et al., 1999). This is based on the known link between exposure to crystalline silica and development of lung diseases, such as silicosis and lung cancer, as a potential long-term health consequence (IARC, 1997). Although the mechanisms through which volcanic ash can initiate a silica-induced disease are not well understood, a recent study has shown that crystalline silica-bearing ash has the potential to activate the NLRP3 inflammasome, a molecular complex that plays a critical role in driving

inflammatory immune responses (Damby et al., 2018). Another commonly investigated potential mechanism is iron-catalysed free radical generation from the ash surface, as discussed in *Section 2.3.4.1*.

2.5.1 Volcanic ash exposure

Personal exposure to volcanic ash is highly influenced by activities undertaken by individuals as well as the general dustiness of the environment; hence concentrations associated with activities such as cleaning, or clearing the roads, may be higher than ambient concentrations, especially for children, since concentrations were found to decrease exponentially with height above the ground (Searl et al., 2002, Horwell et al., 2003b). However, the general community will have lower personal exposures than the ambient levels as people will protect themselves (*e.g.*, staying indoors) during ashfall, overnight and at times of high resuspension. Airborne volcanic ash concentrations are highly dependent upon distance from the volcano, the dynamics of the eruption itself and plume dispersal conditions, and they will dramatically reduce following rain and also through community clean-up efforts. Therefore, it is very difficult to deduce personal exposure, especially as airborne concentrations are rarely known.

Searl et al. (2002) conducted one of the few studies on airborne concentrations of ash during a volcanic eruption sequence, measuring PM_{10} on Montserrat during a period of frequent ashfall (1997-1999) from the Soufrière Hills volcano. They found daily mean concentrations ranging from 0.05-1 mg/m^3 when plentiful ash was in the environment, and 0.02-0.15 mg/m^3 when there was little ash.

2.5.2 Toxicity of volcanic ash

Even though there is a high variability in discrete results of *in vitro* and *in vivo* toxicology assessments for volcanic ash (reviewed by Baxter et al., 2014b, Hillman et al., 2012, and Horwell and Baxter, 2006, Kar-Purkayastha et al., 2012), a general view from the studies, to date, suggests that ash is a particle of limited cytotoxic potential, but various studies have shown that ash can provoke inflammatory

reactions *in vivo* and (pro-)inflammatory reactions *in vitro* (Damby et al., 2016, Horwell et al., 2013, Damby et al., 2013, Lee and Richards, 2004, Damby et al., 2015).

Discerning the components of volcanic ash responsible for any observed toxicity has been difficult due to compositional variability amongst samples and eruptions. In the present study, volcanic ash from Soufrière Hills volcano, Montserrat and Chaitén volcano, Chile were studied. The former posed continuous respiratory health concerns from the onset of the volcano's eruption in 1995 until 2011, due to frequent ashfall events and resuspension of the deposited material. Many of the published toxicological data on the Soufrière Hills ash are reviewed in Baxter et al. (2014b), Horwell and Baxter (2006) and Damby et al. (2016). Overall, these data show that the Soufrière Hills ash is not very reactive *in vitro*. No toxicological studies are published on Chaitén ash but Horwell et al. (*unpublished*) performed a haemolysis test (*i.e.*, lysis of red blood cells) and did not observe any unusual bioreactivity when compared to previous reports for the haemolytic potential of volcanic ash.

2.5.3 Health impact of volcanic ash

A number of clinical and epidemiological studies have examined the health impacts on populations following a volcanic eruption (reviewed in Horwell and Baxter, 2006, Kar-Purkayastha et al., 2012). Similarly to the toxicological studies, the evidence on the incidence of acute respiratory symptoms is variable among the studies. Yet, it has been shown that acute exposure to volcanic ash can trigger and/or exacerbate pre-existing respiratory diseases, such as asthma and bronchitis (Baxter et al., 1983, Baxter et al., 1981). In addition, it has been reported that ash can cause cough, breathlessness, chest tightness as well as irritation to eyes, throat and airways (Searl et al., 2002, Fraunfelder et al., 1983).

Few studies have considered the long-term health consequences of ash exposures, however, no cases of silicosis or other chronic lung disorders have been reported so far (Buist et al., 1986, Cowie et al., 2002, Cowie et al., 2001, Baxter et al., 2014a).

2.6 Volcanic gas-ash interactions

During its 'lifecycle', including transport and dispersal within the volcanic plume during an eruption followed by deposition from the atmosphere, ash is exposed to various physicochemical processes that may change its surface composition and, thus, reactivity in the receiving environment.

Following an eruption, by processes of chemical reaction and adsorption onto the surface of ash particles, volatiles are subsequently scavenged, *i.e.*, removed from the atmosphere and dispersed into the environment, commonly in a form of soluble salt deposits (see *Section 2.3.3.2*) which may impact on ash toxic potential. Thus, the understanding of gas-particle interactions within an eruption plume is highly relevant for health hazard assessment of volcanic ash.

Gas-ash interactions have been previously studied in order to understand the influence they might have on volcanic volatile budgets, surface properties of the ash as well as chemistry and dynamics of a plume (reviewed in Ayris and Delmelle, 2012, Witham et al., 2005). In this thesis, for the first time, the effects of such in-plume processing on the respiratory health hazard of ash particles are investigated (***Chapter 4***).

In-plume scavenging reactions that emplace sulphate and halide deposits on ash surfaces are believed to occur in three temperature-dependent reaction zones above an erupting volcano (*Fig. 2-3*), as originally proposed by Oskarsson (1980). The zones are as follows:

1) the salt formation zone, immediately above the vent, where extremely hot, degassing volatiles form aerosol salt particles which may interact with an aqueous layer on an ash particle and result in adsorption at magmatic temperatures (600 - 1200°C) (Smith et al., 1982, 1983, Taylor and Stoiber, 1973);

2) the surface adsorption zone, where heterogeneous reactions *e.g.*, direct chemical reaction (chemisorption) or physical adhesion (physisorption), between gases (*e.g.*, SO_2, HCl or HF) and exposed ash particle surfaces occur at intermediate

temperatures (300 - 700°C) (Ayris et al., 2014, 2013, Hoshyaripour et al., 2014, Oskarsson, 1980);

3) the condensation zone, where condensation of acids (*e.g.*, H_2SO_4, HCl and HF) forms an aerosol which, in contact with ash, may partially leach and dissolve the particle surface and result in precipitation of salts in the cooling volcanic cloud (< 190°C) (Delmelle et al., 2007, Rose, 1977).

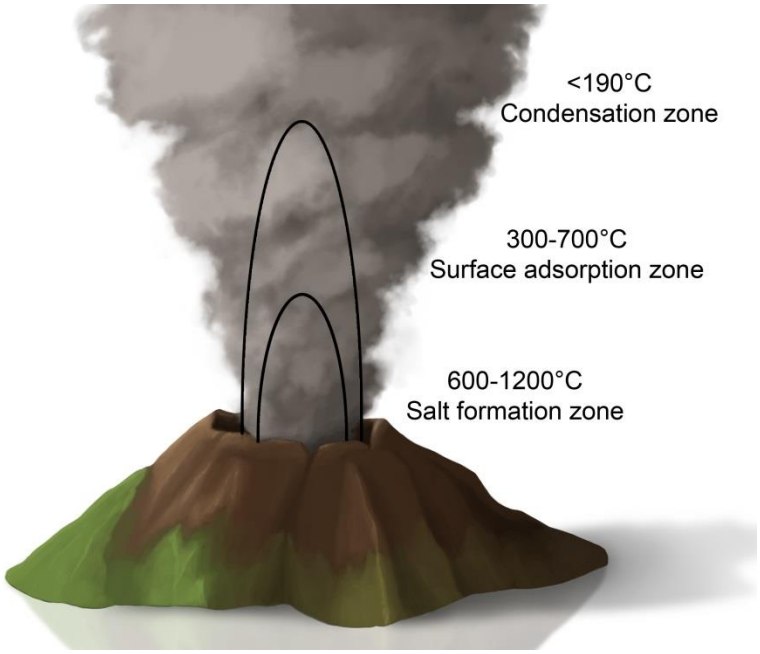

Figure 2-3: Temperature-dependent zones within an eruption plume (a*dapted from Witham et al. (2005), produced by P-Y. Tournigand*).

Current understanding of gas-ash interactions in volcanic plumes is mostly derived from the chemical composition of volcanic ash leachates (Dingwell et al., 2012, Witham et al., 2005), obtained by extracting the soluble materials found on the ash surface. However, leachate data, alone, were found to be insufficient for obtaining an unequivocal understanding due to high spatial and temporal variability in ash leachate compositions (Ayris et al., 2015b). Hence, it has been suggested that additional, more detailed analysis of the surface physicochemical properties of ash are necessary (Ayris and Delmelle, 2012). Most common techniques applied to

investigate surface properties of volcanic ash include high-resolution microscopy and spectroscopy (Durant et al., 2012, Gislason et al., 2011, Delmelle et al., 2007) whereas, several studies have used plume chemical modelling (Textor et al., 2003, Hoshyaripour et al., 2014) to investigate these interactions.

Recently, a novel characterisation method of ash surface chemical properties by heterogeneous titration using reactive probe gases has been used by Maters et al. (2016). This study demonstrated the presence of varying abundance of chemical functional groups on volcanic ash surfaces, including of acidic, basic, reduced and oxidised character, depending on ash composition. Acidic and basic sites are associated with glass and crystalline (aluminosilicate and oxide) components of volcanic ash, and include silanols (Si-OH), siloxanes (Si-O-Si), terminal metal (M) atoms, and oxo (M-O) and hydroxyl (M-OH) groups (Papirer, 2000, Rimola et al., 2013). Redox sites are associated with the presence of species such as transition metal atoms (*e.g.*, iron), which are able to change their oxidation state. Maters et al. (2016) suggest that gas-ash interactions have an important role in modifying ash surface properties, as seen from the differences in probe gas uptake by the natural ash and proxy ash materials (glass), where generally a lower fraction of basic sites (relative to acidic sites) and a higher fraction of oxidised sites (relative to reduced sites) were present on ash compared to glass.

Further, to provide insights into the physicochemical properties and reactivities of volcanic rock surfaces, a sophisticated, experimental setup capable of simulating hot in-plume conditions was developed by Ayris et al. (2015a). It has been used to investigate the uptake of gases such as SO_2 and HCl by ash particles at high temperatures (Ayris et al., 2014, 2013). Previous experiments, where silicate glasses with representative volcanic compositions were exposed to SO_2, demonstrated that the uptake of SO_2 at temperatures above 300°C occurs via rapid adsorption onto reactive Ca-O surface sites, initially forming $CaSO_3$ which is then oxidised, resulting in formation of anhydrite ($CaSO_4$) deposits at the particle surface. This was found to be dictated by diffusion of Ca^{2+} from the particle interior (bulk) to its surface, further sustaining SO_2 adsorption (Ayris et al., 2013). A similar mechanism of diffusion, driven by ion exchange of Na^+ (and other Cl^- reactive

cations) with H^+, appeared to be the HCl uptake mechanism, where the primary reaction product formed during adsorption is halite (NaCl) (Ayris et al., 2014). This system has been applied in the present study to generate salt-laden particles in controlled conditions, which were then used in toxicity assessment (see ***Chapter 4***).

Such in-plume processing, and the associated presence of surface salts, further influences chemical behaviour of ash in the atmosphere during its transport, with regards to its reactivity towards atmospheric trace gases (Maters et al., 2017, Rossi, 2003). This may as well impact the potential of ash to scavenge compounds which originate from anthropogenic sources whilst falling through urban atmospheres.

2.7 Interaction of volcanic ash and urban pollution

Several organic compounds, predominantly hydrocarbons, have been identified in volcanic eruptive materials, including gases and solid products (ash and igneous rocks). The presence of organic compounds in volcanic gases is believed to originate from two processes; they can potentially form by organic (abiotic) synthesis from inorganic compounds (CO_2, CO and H_2) under the catalytic action of rocks and ash (Markhinin and Podkletnov, 1977, 1978, Kolesnikov and Egorov, 1979, Podkletnov and Markhinin, 1981, Zolotov and Shock, 2000) or by decomposition (pyrolysis) of organic material present in the rocks, soil or vegetation on volcanic slopes (Stoiber et al., 1971, Pereira and Rostad, 1983, Pereira et al., 1982, 1980, Isidorov et al., 1990).

While limited in number, other studies have also found molecules such as dioxins and dioxin-like compounds (a type of potentially toxic organic pollutants (WHO, 2010)), polychlorinated biphenyls and polycyclic aromatic hydrocarbons (PAHs) adsorbed on volcanic ash (Lamparski et al., 1990, Stracquadanio et al., 2003, Takizawa et al., 1994), which were shown to originate from anthropogenic sources. These studies suggested that ash can be an effective scavenger and thus, carrier of atmospheric pollutants in the environment (Kozak et al., 2017).

The capacity of mineral dust to uptake atmospheric trace gases such as ozone, sulphur and nitrogen oxides, as well as organic compounds pertinent to urban environments (*e.g.*, PAHs), has been previously studied (*e.g.*, Usher et al., 2003, Falkovich et al., 2004). However, to date, none has looked into the interaction and/or uptake processes of pollutant gases on volcanic ash. The attempt to investigate whether ash interacts with the existing airborne urban pollution is described in ***Chapter 7***.

2.8 Exposure to urban pollutants

Current knowledge about exposure to anthropogenic pollution strongly associates it with adverse health effects, predominantly with increasing or inducing respiratory and cardiovascular diseases, as well as with substantial premature mortality and excess morbidity (Pope et al., 2002, Schulz et al., 2005, Dominici et al., 2006, Pope et al., 2015, Ibald-Mulli et al., 2002, Wichmann and Peters, 2000). Comprehensive reviews of the existing scientific evidence about the health effects of exposure to particulate air pollution have been published by the World Health Organization (WHO) in the *Air Quality Guidelines* (WHO, 2006) and the Technical Report of the project REVIHAAP (WHO, 2013).

In 2012, Global Health Observatory (GHO) data indicated that ambient air pollution was responsible for 3 million deaths worldwide. Furthermore, air pollution is estimated to cause about 8% of COPD deaths, about 17% of respiratory infection deaths and about 25% of lung cancer deaths (see also Straif et al., 2013). Subsequently, WHO's International Agency for Research on Cancer (IARC) concluded that "*outdoor air pollution is carcinogenic to humans, with the particulate matter (PM) component of air pollution most closely associated with increased cancer incidence, especially lung cancer*", classifying both as Group 1 carcinogens (IARC, 2013).

The composition of air pollution and concentrations of exposure can vary dramatically among locations but, generally, human populations resident in urban

areas are particularly affected by high exposures to anthropogenic PM, with the average annual concentrations of PM_{10} ranging from less than 10 to more than 100 $\mu g/m^3$ globally (WHO, 2013, Loomis et al., 2013). Furthermore, most PM_{10} comes from fuel combustion, with vehicles being primary emitters. In this thesis, diesel and gasoline-fuelled vehicle exhaust emission products were used as proxies to evaluate the role of mixing anthropogenic pollutants with volcanic emissions (***Chapter 5*** and ***6***). An exhaustive review of the studies investigating exposures to both diesel and gasoline exhaust focused on their carcinogenic potential has been published by the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (Benbrahim-Tallaa et al., 2012). Relevant information is briefly summarised in the following text.

2.8.1 Diesel exhaust

Exhaust emissions from combustion engines (diesel and gasoline) are a complex mixture made up of solid, condensed and gaseous fractions (Chan et al., 2007), whose composition varies depending on, *e.g.*, engine type and running conditions, type of fuel and lubrication oil (*e.g.*, Popovicheva et al., 2015). Typically, the solid, particle fraction mainly consists of an elemental carbon core and adsorbed organic compounds such as polycyclic aromatic hydrocarbons (PAHs), and small amounts of sulphate, nitrate, metals and metal oxides, and other trace elements derived from the fuel and lubricant (Mayer et al., 2010, Wichmann, 2007, Elliott et al., 1955). The primary gaseous fraction components are nitrogen, water and oxygen (around 99 vol. %), while the rest is made up of potentially toxic gases such as carbon dioxide, carbon monoxide, nitric oxide and nitrogen dioxide (Westerholm and Egebäck, 1994).

Diesel exhaust emissions represent one of the most prevalent anthropogenic pollutants worldwide and their health effects have been extensively investigated. In 2012, IARC classified diesel engine exhaust as a Group 1 carcinogen to humans (IARC, 2012). The most recent, comprehensive review of the current knowledge on diesel exhaust toxic effects was published by Steiner et al. (2016). Overall, the review reports that the biological responses upon interaction with diesel exhaust

are induction of pulmonary oxidative stress and (pro-)inflammation and, partly as their consequence and partly due to separate pathways, the induction of genotoxicity and associated potential for development of lung cancer.

2.8.2 Gasoline exhaust

The main difference between diesel and gasoline engines is their mechanisms of fuel-air mixture preparation and ignition, and the fuels they use. The general view is that diesel engines are a greater contributor in particle emissions (US EPA, 2004), whereas gasoline exhaust contains higher levels of certain gases, such as carbon monoxide (Benbrahim-Tallaa et al., 2012). However, it has been found that vehicles with gasoline engines can also emit substantial quantities of soot-like nanoparticles under certain operating conditions (Mathis et al., 2005, Zhang and McMahon, 2012, Banerjee and Christian, 2017). For example, vehicles with gasoline direct injection (GDI) technology were found to release up to 10^{12} particles/km, exceeding those of current diesel vehicles equipped with filters (Platt et al., 2017, Muñoz et al., 2016, Mohr et al., 2006, Zhang and McMahon, 2012).

Exposure to gasoline exhaust has been associated with lung inflammation, yet, observed pulmonary responses generally seem to be reduced compared with those elicited by diesel exhaust (Benbrahim-Tallaa et al., 2012, Bisig et al., 2015). This has been attributed to the lower concentration (relative to diesel engine exhaust) of produced particles in gasoline exhaust in the studies. Furthermore, little evidence has been found for the carcinogenic effect of gasoline engine exhaust, but such an effect cannot be excluded, thus, it has been classified as *possibly carcinogenic to humans* (Group 2B) (Benbrahim-Tallaa et al., 2012, IARC, 1989).

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Chapter 3

Materials and Methods

Acknowledgments

Bulk ash samples were provided by Claire Horwell (Durham University). Pumice and glass powder of andesitic composition were provided by Paul Ayris (Ludwig-Maximilian-University Munich). Ayris also provided training and support for experiments with the Advanced Ash-Gas Reactor (AGAR). Salt-laden ash and control samples were produced using the AGAR by Ana Casas (Ludwig-Maximilian-University Munich). Separation of the respirable fraction of Soufrière Hills and Chaitén ash was carried out by David Damby (Ludwig-Maximilian-University Munich, now at the USGS California). Damby also provided training and support for separation of samples generated by AGAR.

Training and support for SEM analyses carried out at Durham University were provided by Leon Bowen (Department of Physics), whilst during the secondments at the Adolphe Merkle Institute, University of Fribourg, training and support were provided by Bodo Wilts and Dimitri Vanhecke. XRF analyses of bulk ash were conducted by Nick Marsh (University of Leicester). PSD analyses were conducted with the help of Christopher Rolfe and Samantha Smith at University of Cambridge, Claire Horwell and Neil Tunstall (Department of Geography) at Durham University, and David Damby, Paul Ayris and Ana Casas at Ludwig-Maximilian-University Munich. BET analyses were carried out by David Damby and Ana Casas. Training and support for leachate analyses and ICP-MS measurements at Durham University were provided by Christopher Ottley (Department of Earth Sciences) and Emily Unsworth (Department of Chemistry). IC measurements carried out at Durham University were provided by Amanda Hayton and Kathryn Melvin (Department of Geography).

Training and support for toxicological analyses were provided by Martin Clift (now at the Swansea University), Hana Barošová, Savvina Chortarea, Christoph Bisig, Christoph Geers, Yuki Umehara, Laetitia Haeni, Barbara Drasler and Daniel Hauser (all at the Adolphe Merkle Institute, University of Fribourg). Isolation of monocyte-derived macrophages and dendritic cells was carried out by the members of the BioNanomaterials group.

3.1 Introduction

This chapter includes detailed a description of the main analytical and experimental techniques (repeatedly) used across the studies in this thesis, including isolation of the respirable ash fraction, physicochemical characterisation of ash, description of the 3D *in vitro* multicellular model of the lungs and biochemical analyses used to assess cellular response to particle treatments. The methods which appear in only one chapter are included within that chapter.

3.2 Volcanic ash sample selection and source

The interactions with anthropogenic pollution studies (***Chapter 5*** and ***6***) focus on a small selection of natural volcanic ash samples, selected primarily for their pristine nature (erupted, transported and deposited into a relatively non-polluted environment, collected prior to rainfall) but also taking into account the amount of respirable (sub-4 μm) ash in the sample, to ensure that sufficient material could be isolated from the bulk and used for toxicological experiments. Using samples in their pristine (*i.e.*, non-weathered) condition was important to ensure the ash had preserved leachable and reactive surface elements that would have otherwise been naturally leached into the environment, *e.g.*, during rainfall (Hinkley, 1987, Witham et al., 2005).

Finding samples of ash which meet these criteria is challenging. Chosen samples represent intermediate (andesitic) (*Section 3.2.1*) and felsic (rhyolitic) material (*Section 3.2.2*) as these magmatic compositions tend to generate highly explosive eruptions and, hence, fine-grained ash (***Chapter 2***, *Section 2.3.3.1*). However, a basic, basaltic sample could not be obtained. In general, it is known that basaltic volcanoes tend to produce relatively coarse ash compared to more explosive eruptions (Horwell, 2007) and no pristine basaltic ash could be sourced with sufficient respirable material. Selected samples were sourced from the collection of Dr Claire Horwell (Durham University).

For the interactions with volcanic gases experiments (see ***Chapter 4***), samples of ash were generated by fragmenting Soufrière Hills pumice in order to obtain

fresh, reactive particle surfaces (which had not previously been exposed to gases in a volcanic plume) for further experiments with the Advanced Ash-Gas Reactor (AGAR; Ayris et al., 2015). In addition, a glass powder of andesitic composition was used in these experiments as a proxy for volcanic ash. The production of these samples and experiments with the AGAR are described in detail in ***Chapter 4***.

3.2.1 Soufrière Hills, Montserrat

The Soufrière Hills volcano, Montserrat erupted andesitic lava from July 1995 and, since then, there have been five eruptive phases and five pauses. The latest phase of eruptive activity ended in February 2010 and, since then, a slow, steady deformation of the ground surface around the volcano has been observed, associated with magma recharge (*www.mvo.ms*[1]). Throughout the eruption, the volcano posed a continuous respiratory hazard due to the substantial amount of respirable ash released by frequent lava dome collapses (a particular style of eruption in which a mound of viscous lava, that has piled up over the vent, collapses and fragments), and the crystalline silica that the ash contains (Horwell et al., 2014, Baxter et al., 1999). As reported in Baxter et al. (2014), the ash has been extensively studied over time and well characterized (Horwell, 2007, Horwell et al., 2007, 2003a, 2003b, 2014) for respiratory hazard assessment.

The ash sample used in this study was generated in a dome-collapse event on 12 July 2003 and was collected 4 km from the vent on the day of the eruption. The bulk sample's physicochemical characteristics can be found in previous literature under different sample codes, as follows: *Soufrière Hills, Montserrat '03* in Horwell et al. (2007), *Soufrière Hills, Montserrat 2003* in Horwell (2007), *Mon12/7/03* in Horwell et al. (2010) and *MBA12/7/03* in Horwell et al. (2013). Briefly, the ash is rich in crystalline silica (~12 wt. % of the bulk ash is cristobalite), and is considered fine-grained for ash, with ~11.5 vol. % of the bulk sample being sub-4 µm diameter (Horwell, 2007).

[1] 22nd Report of the Scientific Advisory Committee (SAC) on Montserrat Volcanic Activity, Part II: Full Report, issued on February 25th, 2018.

3.2.2 Chaitén, Chile

Chaitén volcano, Chile, erupted in 2008 which resulted in the significant fallout of rhyolitic ash in Argentina (Martin et al., 2009). The ash posed a potential respiratory health hazard due to the fact that it contains substantial quantities of respirable particles including crystalline silica. The amount of cristobalite increased substantially after dome growth and collapse began 3 months into the eruption (Horwell et al., 2010) but the initial explosive phase also generated ash containing cristobalite, either through disruption of the pre-existing obsidian dome (Horwell et al., 2010) or due to in-plume generation of nano-silica fibres, a hypothesis proposed by Reich et al. (2009).

The sample used in this study was deposited from one of the early explosive eruptions which occurred on 2 May 2008 and was collected 80 km away from the source, in the Patagonian Argentine province of Chubut, on 8 May 2008 (Horwell et al., 2010). This sample contains substantially less crystalline silica (~3 wt. % of the bulk ash is cristobalite) than the Soufrière Hills volcano sample (3.2.1), but is similarly fine-grained, with ~12 vol. % sub-4 µm material (as *Chai_03* in Horwell et al., 2010).

3.3 Volcanic ash sample preparation

Isolation of respirable (sub-4 µm) fractions from the bulk samples was a crucial step in ensuring that the toxicological experiments were performed with particles that were biologically relevant, *i.e.*, that their size corresponds to particles that have potential to enter into the deep lung (alveolar region) (see ***Chapter 2***, *Section 2.3.3.1*).

3.3.1 Isolation of the respirable fraction

Respirable fractions of volcanic ash samples were isolated using a Sioutas cascade impactor (*SKC Inc.*, USA) and Leland Legacy sample pump (*SKC Inc.*, USA) attached to a gravitational separation chamber (*Fig. 3-1*).

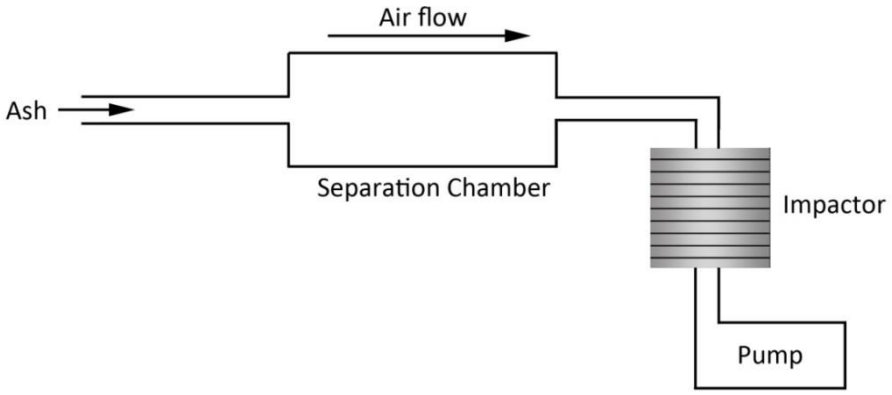

Figure 3-1: Schematic of the set up used to separate the fine fraction of volcanic ash.
Produced by P-Y. Tournigand.

Ash was introduced *via* a tube into an airstream established by operating the pump at a constant flow rate of 5 L/min. Aerosolised particles then entered a separation chamber where particles above a theoretical spherical aerodynamic diameter of 5 µm sedimented in accordance with Stoke's law, calculated for a particle density of 1.0 g/cm^3 in the system. These parameters have been empirically observed to produce an appropriate respirable-sized fraction in this set up (Damby et al., 2016). Remaining airborne particles were then sampled by the impactor, which was assembled without impaction stage filters to enable sample recovery. Size-fractionated samples were then collated for use in characterisation and toxicity assays.

Separation of the respirable fraction from the sedimented ash was conducted upon the same sub-sample on two or three different occasions and then combined in order to maximise the recovery of respirable material needed for the studies.

3.4 Physicochemical characterisation of volcanic ash

This section contains a description of the methods used for physicochemical characterisation of samples, to define the bulk composition and the parameters relevant to respiratory toxicity (see ***Chapter 2***, *Section 2.3.3*).

3.4.1 Ash morphology

Secondary electron (SE) detection by scanning electron microscopy (SEM) was used to image morphology and surface textures of ash samples, mainly to identify differences among samples. Imaging of samples was carried out on a Hitachi SU-70 (FEG) SEM in the GJ Russell Microscopy Facility, Department of Physics, Durham University, unless otherwise stated.

Samples were prepared for imaging by spreading particles across a polycarbonate sheet using a cotton bud and adhering the sheet to an aluminium stub using a carbon sticky pad. Samples were sputter coated with gold/palladium to a thickness of ~30 nm to prevent charging of the particles under the beam. Operating voltage, working distance and magnifications for individual images varied; therefore these are either recorded on the image or noted in the figure caption.

3.4.2 Magmatic composition

To identify and quantify the major and trace elemental composition of the bulk ash samples X-ray fluorescence (XRF) was used. Sample compositions were used to confirm previously-reported magmatic compositions of samples on a total alkali versus silica (TAS) plot. XRF analysis was performed at the Department of Geology, University of Leicester, UK using a PANalytical Axios Advanced PW4400 XRF spectrometer.

Major elements were determined on fused glass beads prepared from ignited ash powders mixed with a fluxing agent (80 % Li metaborate, 20 % Li tetraborate) in a 1:5 ratio. Results were re-calculated to include loss on ignition (LOI) value and expressed as component oxide weight percent. Trace elements were analysed on 32 mm diameter pressed powder pellets produced from mixing 7 g of fine ground sample powder with 12-15 drops of a 7 % poly(vinyl alcohol).

3.4.3 Particle size distribution

In this thesis, ash particle size was measured using laser diffraction based on geometric particle diameter which refers to the equivalent diameter of a sphere and is expressed as volume % (vol. %). Even though not directly comparable to the aerodynamic diameter, size fractions determined in such way give an insight into the potential of particles to deposit in the respiratory tract.

The cumulative particle size distributions of bulk ash samples were measured using laser diffraction at the Department of Geography, University of Cambridge, UK. Analyses were conducted using a Malvern Mastersizer 2000 with ultrasonics, following a standard operating procedure of 10 s measurement time, obscuration of 5–20 % and pump speed 2000 rpm. Data were analysed according to the Mie theory of light scattering (Mie, 1908). For all data, the refractive index was set to 1.63 and absorption to 0.1, as outlined in Horwell (2007). Results are reported as the average of 3 recorded consecutive runs of each sample. Data are obtained as volume percentages, and are later converted into cumulative vol. %.

To obtain precise data on the health-pertinent fractions of bulk samples, the data points (for the binned data, according to the standard operating procedure of the instrument) were then interpolated to give cut-offs at 1, 2.5, 4 and 10 µm.

3.4.4 Specific surface area

The specific surface area of ash was determined according to the Brunauer-Emmett-Teller (BET) method (Brunauer et al., 1938) of nitrogen adsorption, where the amount of adsorbed gas corresponds to the surface area of a solid. Samples were dried overnight at 105 °C prior to analysis. Experiments were carried out using a Gemini III 2375 surface area analyser at Department of Earth and Environmental Sciences, Section for Mineralogy, Petrology and Geochemistry, Ludwig-Maximilian-University Munich, Germany.

Insufficient amount of respirable material was recovered from the separation (*Section 3.3.1*) to perform BET analysis of all ash or volcanic glass samples used in

toxicity studies. Due to this, the dose metric used was particle mass. This represents one of the limitations of the current study, considering it has been suggested that the surface area is a better-suited dose metric for predicting the adverse health outcomes of particle exposures (Duffin et al., 2007), compared to particle mass, especially in the case of biopersistent particles (Schmid and Stoeger, 2016).

3.4.5 Surface soluble species

Freshly erupted ash may contain a range of (potentially-toxic) soluble elements such as As, Cd, Cr, Cu, F, Fe, Mn, Ni, Pb, V and Zn, which may be released either rapidly or more slowly upon contact with water or body fluids (Witham et al., 2005) and may be harmful (*e.g.*, Costa and Dreher, 1997, Adamson et al., 2000, Ghio and Devlin, 2001). These chemical species are commonly quantified using leachate methods (Stewart et al., 2013).

At the beginning of the present project, as part of extensive training in leachate analysis carried out in the Geochemistry Facility at Durham University, the concentrations of soluble elements from the bulk ash used in this thesis (*Sections 3.2.1* and *3.2.2*) were measured by leaching samples in water and acid (*Section 3.4.5.1*). Water leach reflects the dissolution of readily-soluble compounds adsorbed onto the ash surfaces, whereas the acid leach gives an insight into the additional solubility of elements under acidic conditions (*e.g.*, in the gastric system). Following these initial tests, samples were reanalysed (to include two complementary water-leach tests at different solid/liquid ratios) according to the rapid-response protocol for the assessment of hazards from leachable elements in volcanic ash, as proposed by the International Volcanic Health Hazard Network (IVHHN), and concurrently with an international round-robin exercise for validation of the new laboratory protocol (Stewart et al. 2018, *manuscript in preparation*). Finally, this work has led to the experiments assessing the viability of using a simulated lung fluid method (*Section 3.4.5.2*) for a rapid assessment of potential hazards from leachable elements in volcanic ash in the lungs.

3.4.5.1 Water and acid leach

The bulk natural ash samples were leached with deionised water, and separately, with nitric acid (3.5 % HNO_3) at ratio of 1:20 (g dry weight ash to mL extractant) in at least three replicates. During the extraction, the samples were agitated using a benchtop shaker for 1 hour at room temperature. Subsequently, samples were centrifuged for 10 min (~4500 g) and supernatants were filtered through 0.45 µm filters for cation analysis. The concentrations of major (Ca, Mg, Na, K) and trace elements (*e.g.*, Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) in collected extracts were measured by inductively coupled plasma mass spectrometry (ICP-MS) at the Department of Earth Sciences, Durham University. The results were expressed in mg per kg of dry ash weight (ppm).

During the round-robin exercise, the samples were reanalysed to include two complementary tests at ratios of 1:20 and 1:100 (g dry weight ash to mL water), as per the protocol (Stewart et al., 2013), and to perform both cation and anion analysis of water leachates. The extraction procedure was the same as described above. The sub-samples of supernatants for anion analysis were filtered through 0.2 µm filters. Anion analysis (Cl^-, F^-, SO_4^{2-}, PO_4^{3-}, NO_3^-) of ash leachates was carried out at the Department of Geography, Durham University using ion chromatography (IC).

Since no direct correlation to other results of toxicological assessment in the thesis was done, these data are reported in ***Appendix 1***. The experiences from this particular work, however, contributed to the mentioned round-robin exercise (Stewart et al. 2018, *in prep.*) and the subsequent leachate analyses (*described below*).

3.4.5.2 Simulated lung fluid leach

The simulated lung fluid (SLF) mimics the composition of the interstitial lung lining fluid and is commonly used to assess the biosolubility and biodurability of a wide range of materials in the lungs (*e.g.*, Plumlee and Morman, 2011, Wiseman, 2015, Kastury et al., 2017, Caboche et al., 2011). There are varying formulations of SLF

that have been used but, generally, the solution represents a complex mixture of reagents, including electrolytes and organic constituents (Gamble's solution; *e.g.*, Colombo et al., 2008, Midander et al., 2007, Stopford et al., 2003). Briefly, the solution has a near neutral pH (7.4) and consists of a balance of cations (Na, K, Ca, Mg) and anions (HCO_3^-, Cl^-, HPO_4^{2-}, SO_4^{2-}) with acetate ($H_3C_2O_2^-$) and citrate ($H_5C_6O_7^{3-}$), which substitute organic acids and proteins, respectively (Gamble, 1967, Moss, 1979) (*Table 3-1*).

Table 3-1: Chemical composition of simulated lung fluid – Gamble's solution (Moss, 1979).

Composition	Gamble's solution (g/L)
Magnesium chloride	0.095
Sodium chloride	6.019
Potassium chloride	0.298
Disodium hydrogen phosphate	0.126
Sodium sulphate	0.063
Calcium chloride dihydrate	0.368
Sodium acetate	0.574
Sodium hydrogen carbonate	2.604
Sodium citrate dehydrate	0.097

To date, there are no published data on ash leachates in SLF, but some preliminary results (Carol Stewart, Massey University and Suzette Morman, USGS, *personal communication*) have shown that the measurement of concentrations of the most abundant major cations present on the surface of volcanic ash, such as Ca, K and Na, by inductively coupled plasma mass spectrometry (ICP-MS) is not 'straight-forward' due to the matrix effects, *i.e.*, a type of measurement interference caused by dissolved ions (matrix constituents) in the solution. Since these ions are already present in the SLF in high concentrations (*i.e.*, high background values), this can cause a signal reduction or poor precision during measurement, thus causing difficulties in determining concentrations leached from

the ash. Furthermore, the reagents for preparing the SLF have to of a high grade/purity in order to keep the background levels at low concentrations.

Hence, prior to conducting the research, it was first investigated whether other media could act as proxies for SLF. Deionised water, saline and cell culture medium were chosen as analogues. Deionised water represents a simple analogue solution for SLF; its main advantage is the lack of interfering elements during ICP-MS analysis. For these reasons, it has been commonly used in the past in inhalation bioaccessibility tests (reviewed in Caboche et al., 2011), but also because some studies have suggested that it is the 'water-soluble' compounds that may be associated with toxic effects in the lungs (Benson et al., 1986, Costa and Dreher, 1997, Oller et al., 2009). Saline solution (prepared by dissolving sodium chloride (NaCl) in deionized water) was also chosen since NaCl is the major constituent of an SLF (*Table 3-1*) and has a higher ionic strength than water; therefore it represents a 'simplified SLF'. Finally, cell culture medium (RPMI 1640, *Thermo Fisher Scientific*, UK), a liquid designed to support the growth and maintenance of cells, was used as a proxy for a more complex SLF solution, constituting inorganic salts (NaCl, KCl, $Ca(NO_3)_2$, $MgSO_4$, $NaHCO_3$, Na_2HPO_4), amino acids, vitamins and other components (***Appendix 1***). The cell medium is internationally commercially available and closely resembles the physiological and chemical properties of lung fluid.

For these experiments, bulk Soufrière Hills ash (SHV; *Section 3.2.1*) was leached with deionised water, saline solution (NaCl, 6 g/L) and cell culture medium (RPMI) (*Fig. 3-2*). The ash was leached at a ratio of 1:100 (g dry weight ash to mL extractant) in two replicates (except n=1 for RPMI), each with a contact time of 1 hour at room temperature and agitated using a benchtop agitator. Subsequently, supernatants were filtered through a 0.45 µm filter for cation analysis. The concentrations of a range of elements (Li, B, Al, K, Ti, Cr, Fe, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Ba, Pb) in collected extracts and blank solutions were measured by ICP-MS, to assess whether the amounts of extractable elements are similar among different proxies for SLF. Samples were diluted 1:10 prior to the measurement. The results were expressed in mg per kg of dry ash weight (ppm).

Separately, an additional test was conducted to assess whether the extraction-time of 1 h was appropriate (for use with regards to the protocol for rapid hazard assessment), instead of 24 h, which is commonly used in SLF leachates. For this purpose, the rate of dissolution was measured with SHV ash leached in NaCl, as described above.

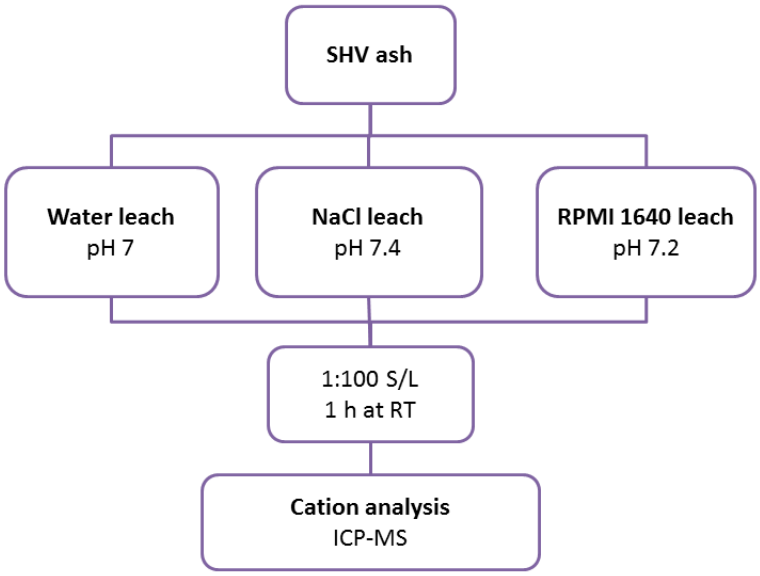

Figure 3-2: Scheme of the leachate comparison exercise using different simulated lung fluid proxy solutions: deionized water, saline solution (NaCl) and cell culture medium (RPMI), at comparable pH.

The outcome of this exercise was that the only element that could be measured in all three solvents was Mn, with its concentrations comparable among the three different extracts (*Table 3-2*, *Fig. 3-3*). Some minor elements (Li, Co, Cu) were also comparable between water and RPMI (*Table 3-2*). Other elements were either i) already present in the blank solutions in substantial quantities, notably K in both NaCl and RPMI, hence, making the measured concentrations of samples less reliable since the measured concentrations were very similar to the blanks, or ii) they were below the detection limit of the method, which means that either the element was not dissolved and thus, detected, or the concentrations measured in samples were, again, too similar to the blank concentration (*Table 3-2*).

From these data, no differences in the extraction efficiency among the solvents could be observed. Additional ash samples (from Chaitén (*Section 3.2.2*), Kelud and Sakurajima volcano) were leached in water and NaCl, in one replicate each, and analysed concurrently as described above, to assess whether the observations could be confirmed. Again, only Mn could be measured with confidence, in Kelud and Sakurajima ash leachates, and the concentrations were comparable (*Table 3-2*, *Fig. 3-3*).

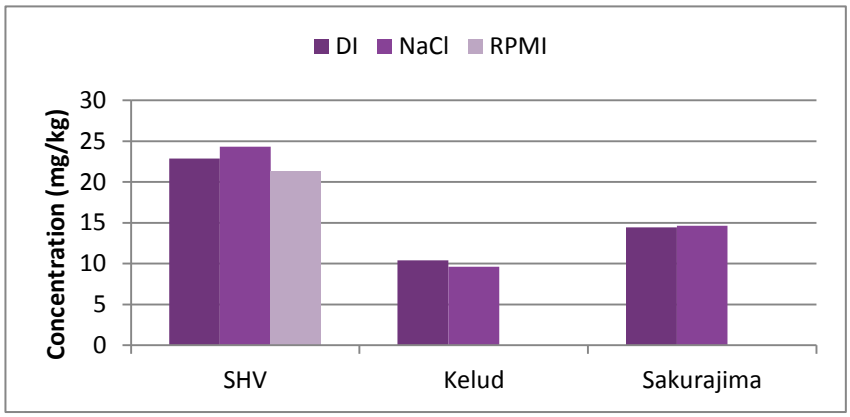

Figure 3-3: Mn concentrations (mg/kg) measured after 1 h extraction of SHV, Kelud and Sakurajima ash samples at 1:100 S/L ratio in deionized water (DI; n=2 for SHV, n=1 other samples), saline solution (NaCl; n=2 for SHV, n=1 other samples) and cell culture medium (RPMI; n=1).

The results of the NaCl leach time-series showed that the concentrations of measurable, soluble elements are in good agreement between the different time-points, indicating that the majority of readily-soluble species are leaching from the particle surface within the first hour of the experiment (*Fig. 3-4*).

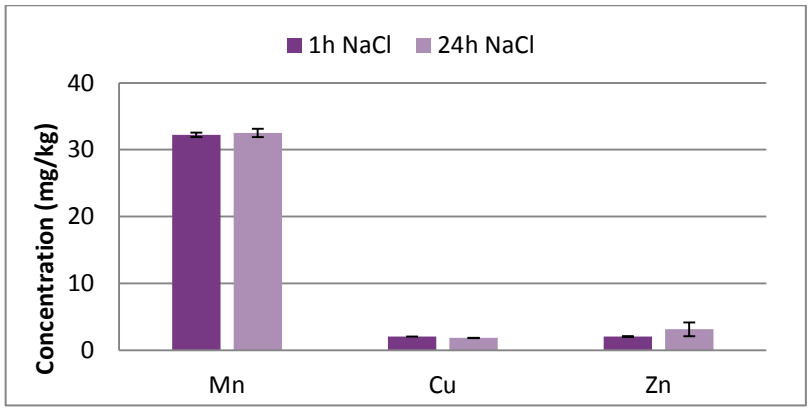

Figure 3-4: Comparison of the sodium chloride-extractable elements at two time-points.

Table 3-2: Element concentrations (mg/L) measured after 1 h extraction of SHV ash at 1:100 S/L ratio in deionized water (DI; n=2), saline solution (NaCl; n=2) and cell culture medium (RPMI; n=1). Values reported are raw data as measured by ICP-MS. DL = detection limit of the method, determined as average of measured blank + 3 SD (= standard deviation) of the blank. Values in green are those above the DL. One replicate of NaCl blank (Blank NaCl_2) was potentially contaminated.

	Element concentration (ppb)																	
Sample	**Li**	**B**	**Al**	**K**	**Ti**	**Cr**	**Fe**	**Mn**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Mo**	**Cd**	**Ba**	**Pb**
Blank DI_1	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	3.52	0.00	0.00	0.00	0.00	0.00	0.00
Blank DI_2	0.01	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	2.14	0.00	0.00	0.00	0.00	0.00	0.00
SHV_DI_1	0.15	0.00	4.40	42.25	0.01	0.00	0.00	23.04	0.15	0.02	2.08	2.47	0.00	0.00	0.00	0.01	0.11	0.00
SHV_DI_2	0.12	0.00	3.60	37.63	0.02	0.00	0.00	22.64	0.14	0.02	1.87	1.97	0.00	0.00	0.00	0.01	0.03	0.00
ChV_DI	0.16	0.30	4.43	14.29	0.00	0.00	0.00	4.58	0.00	0.01	0.04	1.12	1.25	0.00	0.02	0.00	0.18	0.00
Kelud_DI	0.08	0.00	12.89	10.36	0.00	0.00	0.00	10.38	0.03	0.02	4.91	1.81	0.00	0.00	0.00	0.00	1.53	0.00
Sakurajima_DI	0.20	0.00	108.20	0.00	0.01	0.00	0.00	14.42	0.64	0.41	4.87	1.86	0.00	0.00	0.00	0.06	0.05	0.00
Blank_DI (n=2)	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.83	0.00	0.00	0.00	0.00	0.00	0.00
SD	0.00	0.07	0.00	0.63	0.03	0.01	1.48	0.00	0.00	0.01	0.01	0.98	0.02	0.03	0.00	0.00	0.00	0.00
DL	**0.02**	**0.20**	**0.01**	**1.88**	**0.09**	**0.04**	**4.45**	**0.01**	**0.00**	**0.04**	**0.02**	**5.76**	**0.07**	**0.08**	**0.00**	**0.00**	**0.01**	**0.01**
Blank RPMI_1	0.06	0.90	0.00	18830.00	0.62	3.10	0.00	0.01	0.03	0.20	2.05	1.96	0.00	0.03	0.02	0.00	0.03	0.00
Blank RPMI_2	0.06	1.96	0.00	19570.00	0.64	3.61	0.00	0.02	0.03	0.22	2.37	2.74	0.00	0.85	0.04	0.00	0.04	0.00
SHV_RPMI	0.17	0.09	0.00	19190.00	0.55	3.26	0.00	21.36	0.17	0.24	4.05	4.14	0.00	2.15	0.06	0.01	0.00	0.00
Blank_RPMI (n=2)	0.06	1.43	0.00	19200.00	0.63	3.36	0.00	0.01	0.03	0.21	2.21	2.35	0.00	0.44	0.03	0.00	0.04	0.00
SD	0.00	0.75	0.04	523.26	0.01	0.36	4.35	0.01	0.00	0.01	0.23	0.55	0.08	0.58	0.01	0.00	0.00	0.00
DL	**0.07**	**3.68**	**0.11**	**20769.78**	**0.66**	**4.43**	**13.04**	**0.04**	**0.03**	**0.25**	**2.89**	**4.00**	**0.24**	**2.19**	**0.06**	**0.01**	**0.04**	**0.01**
Blank NaCl_1	0.07	3.26	2.66	215.90	0.29	0.16	0.00	0.01	4.37	0.07	2.89	3.98	0.00	2.55	0.03	0.00	2.36	0.02
Blank NaCl_2	0.32	7.81	286.70	282.40	0.41	13.56	46.04	1.80	4.68	88.21	6.22	29.00	0.00	4.36	0.83	0.01	4.27	0.96
SHV_NaCl_1	0.18	3.44	0.60	283.90	0.25	0.11	0.00	24.01	4.50	0.30	7.01	4.06	0.00	6.78	0.06	0.01	2.06	0.00
SHV_NaCl_2	0.17	3.24	0.00	294.10	0.26	0.06	0.00	26.46	4.52	0.10	9.06	5.97	0.00	8.78	0.08	0.01	2.04	0.00
ChV_NaCl	0.17	3.94	2.88	279.30	0.27	0.05	0.00	3.48	2.88	0.09	10.12	1.02	1.02	11.10	0.09	0.00	3.30	0.00
Kelud_NaCl	0.13	3.18	0.31	291.70	0.34	0.02	0.00	10.51	4.17	0.10	13.95	3.88	0.00	12.27	0.08	0.01	7.25	0.00
Sakurajima_NaCl	0.26	2.82	38.56	252.60	0.28	0.00	0.00	15.53	5.33	0.51	19.09	2.92	0.00	13.96	0.07	0.08	2.37	0.02

Earlier studies argued that simple leaching solutions, such as water, are not 'physiologically based' and thus, not representative of pulmonary exposure (reviewed in Kastury et al., 2017). It is also believed that a water leach may underestimate the pulmonary bioaccessibility of metal components due to the absence of proteins and organic compounds (that are represented by citrate, glycine and cysteine in an SLF), which act as chelating agents and may dissolve otherwise insoluble compounds (Caboche et al., 2011, Pelfrêne et al., 2017). However, the majority of studies focus on the determination of potentially toxic metals such as Pb, Zn, Cd, Co, Cu, from various ambient particles, and do not report the leachable concentrations of cations such as Ca, Na, K and Mg. These elements are the main constituents of ash surface coatings (***Chapter 2***, *Section 2.3.3.2*), and in the present study, the research in ***Chapter 4*** aimed to assess whether these salt coatings may potentially impact ash respiratory toxicity *in vitro* through leaching in contact with cell surfactant (*i.e.*, lung fluid *in vivo*). Thus, to assess their dissolution, samples would ideally be leached in a SLF.

Since it was shown during the leaching test that it is challenging to measure ash-leachable K in these still relatively 'simple SLF' solutions (NaCl and RPMI), due to the high background values and associated difficulties with the measurement, as mentioned earlier in this *Section*, it was decided not to proceed with conducting leachate experiments using SLF. Instead, for the purpose of research in ***Chapter 4*** (see *Section 4.2.5*), a water leach was used as a simple analogue and acceptable indicator, accompanied with geochemical modelling, to investigate which species might dissolve upon contact with lung fluid. This was reasoned by consideration that water should leach such elements as rapidly as lung fluid. However, based on the test performed here, the viability of SLF for use in the assessment of hazards from minor elements present in volcanic ash cannot be disregarded, but requires further experimental work.

3.5 In vitro toxicology

3.5.1 Triple cell co-culture

In vitro experiments throughout this study were performed using an established multicellular human lung model composed of three cell types mimicking the human alveolar epithelial tissue barrier (Rothen-Rutishauser et al., 2005, Blank et al., 2007, Rothen-Rutishauser et al., 2008), cultured at the air-liquid interface (ALI). This was the first time such an approach was used, as all previous *in vitro* studies performed on volcanic ash have used monoculture cell models, immersed in cell medium (see discussion in ***Chapter 5***).

The model consists of a layer of lung epithelial cells, monocyte-derived macrophages and monocyte-derived dendritic cells (*Fig. 3-5*). Its 3D structure and intercellular signalling (*i.e.*, communication between different cells) represent a more physiologically relevant model, as it more closely mimics the real situation in alveoli rather than using monocultures or monocultures and macrophages only.

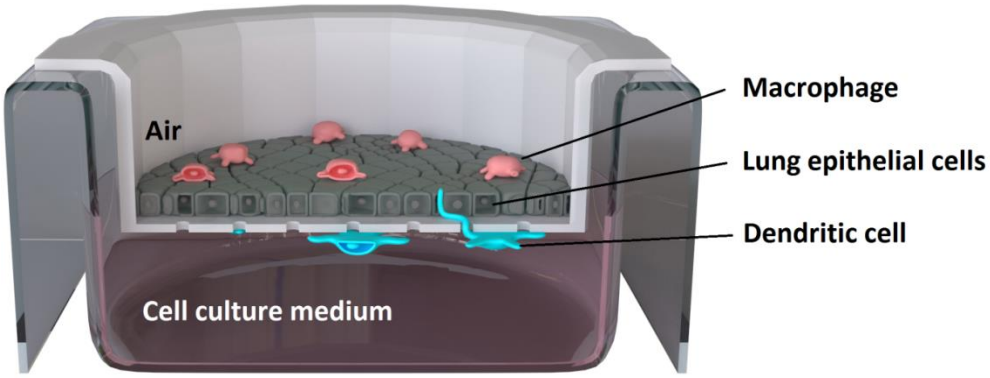

Figure 3-5: Triple cell co-culture composed of epithelial cells, monocyte-derived macrophages and dendritic cells. The cells are exposed to air in the upper chamber and to cell culture medium in the lower part. Adapted from Fytianos et al. (2016).

The human alveolar type II-like epithelial cell line A549, which originates from human lung carcinoma (Lieber et al., 1976), has key biological properties of alveolar

epithelial type-II cells, such as specific structural characteristics (*e.g.,* tight junctions), and is being widely used in *in vitro* models (Lehmann et al., 2010). Macrophages are specialised cells originating from blood monocytes involved in the detection, phagocytosis and destruction of pathogens. They initiate inflammation by releasing inflammatory cytokines that activate other cells. Dendritic cells are cells responsible for the initiation of adaptive immune responses and hence function as the 'sentinels' of the immune system (Lambrecht and Hammad, 2009).

Epithelial cells (A549) were cultured on polyethylene terephthalate (PET) membrane 6-well inserts (4.2 cm^2 growth area, 3.0 µm pore size; *BD Falcon™ Cell Culture Inserts, BD Biosciences,* USA) at a density of 23.8 x 10^4 cells/cm^2 and maintained at 37 °C and 5 % CO_2. They were grown for 5 days prior to the addition of immune cells to form a co-culture.

Human blood monocytes were isolated from buffy coats provided by the Transfusion Blood Bank (*Blutspendedienst SRK Bern AG*, Switzerland) with the adaptation of using $CD14^+$ magnetic beads (CD14 MicroBeads, *Miltenyi Biotec*, Germany) (Steiner et al., 2012) and differentiated intothe monocyte-derived macrophages (MDM) and dendritic cells (MDDC), as described in Lehmann et al. (2010). MDM and MDDC were then added on the apical (1.2 x 10^4 cell/cm^2) and the basal (6.0 x 10^4 cell/cm^2) side of the insert containing the A549 layer, respectively.

After 24 h incubation under submerged conditions, in complete RPMI 1640 cell medium (cRPMI; *Sigma-Aldrich*, Switzerland; supplemented with 1 % L-Glutamine, 1 % Penicillin/Streptomycin and 10 % fetal bovine serum), the triple-cell co-culture was transferred to the ALI for a period of 24 h prior to exposures.

3.5.2 Cell culture exposures

All exposures of cell cultures to volcanic ash (***Chapter 4***, ***5*** and ***6***) were conducted, for the first time in *in vitro* studies of the respiratory hazard of volcanic ash, by nebulising the respirable fraction of ash for direct deposition onto the cell cultures at the ALI (as described in *Section 3.5.2.1*). This method has enabled application of volcanic ash to cells in its pristine, dry state. This represents a more realistic

scenario in comparison to all previous studies which used suspended ash in cell medium, *i.e.*, a pre-mixed sample. The method was initially set up by conducting a dose-dependent analysis and subsequent dose-response analysis (*Sections 3.5.2.2* and *3.5.2.3*) to determine the optimal dose to use for volcanic ash in the cell exposure experiments.

3.5.2.1 Volcanic ash exposure system

Respirable volcanic ash was nebulised, dry, directly over the cells at the ALI using a dry powder insufflator (Model DP-4; *PennCentury Inc.*, USA), which has been previously used in studies of different nanoparticles (Blank et al., 2006) (*Fig. 3-6*). The device is designed to produce a cloud of fine particles from the end of a small-diameter delivery tube, in order to administer a dry powder and for various *in vitro* applications. The body of the device is made of chemically-resistant PEEK™ (polyether-etherketone) parts and silicone valves, whilst the delivery tube is made of stainless steel and has a 120-degree bend.

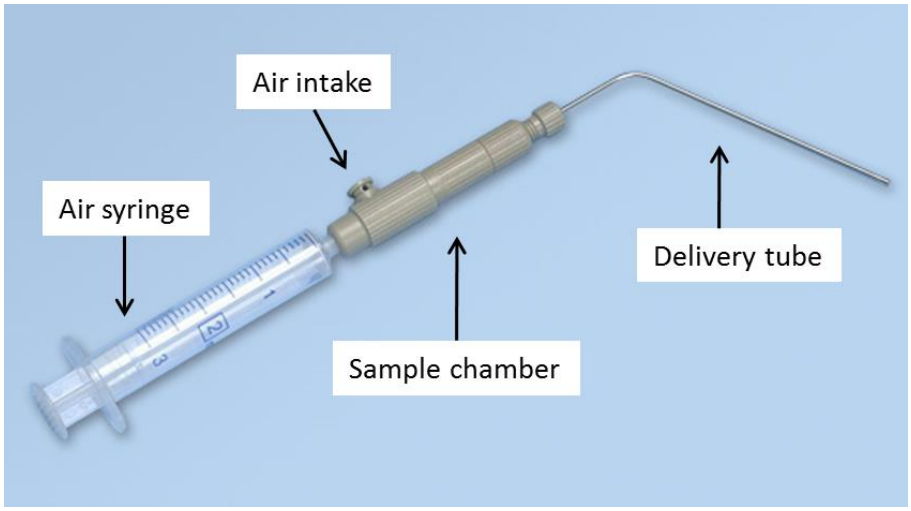

Figure 3-6: Penn-Century Dry Powder Insufflator™ Model DP-4 used to nebulise dry volcanic ash for direct deposition over the cell cultures. Adapted from device literature (*Penn-Century, Inc.*, 2012).

The ash was loaded into a sample chamber and then pushed through the device by small pulses of air administered to the device using a 10 mL commercial syringe. The ash was discharged as a cloud from the end of a delivery tube and, in

this way, nebulised over the cell culture plate located below the delivery tube within a closed nebulisation chamber. The chamber was made of polystyrene and covered with aluminium foil on the inside to avoid particles being electrostatically attracted and sticking to the chamber walls.

The quantification of deposited material was monitored by a quartz crystal microbalance (QCM; with a detection limit of 90 ng/cm^2, AT-cut quartz, 5 MHz resonance frequency *Stanford Research Systems*, USA) (*Fig. 3-7*) also located within the nebulisation chamber next to the wells, thereby allowing for an estimation of the deposited mass. Specifically, as material settles onto the QCM, the frequency of the crystal changes (ΔF) such that, with increasing deposited mass, the resonant frequency of the crystal linearly decreases (Lenz et al., 2009). Calculated from the recorded frequency values before and after deposition of material, this ΔF value (Hz) is converted to deposited mass per area (µg/cm^2) using the Sauerbrey equation (*Eq. 3-1*),

Equation 3-1: Sauerbrey equation.

$$\Delta m = -\Delta F / C_f$$

where C_f is equal to 56.6 Hz cm^2/µg (at room temperature) for a 5 MHz, AT-cut, α-quartz crystal (Lenz et al., 2009).

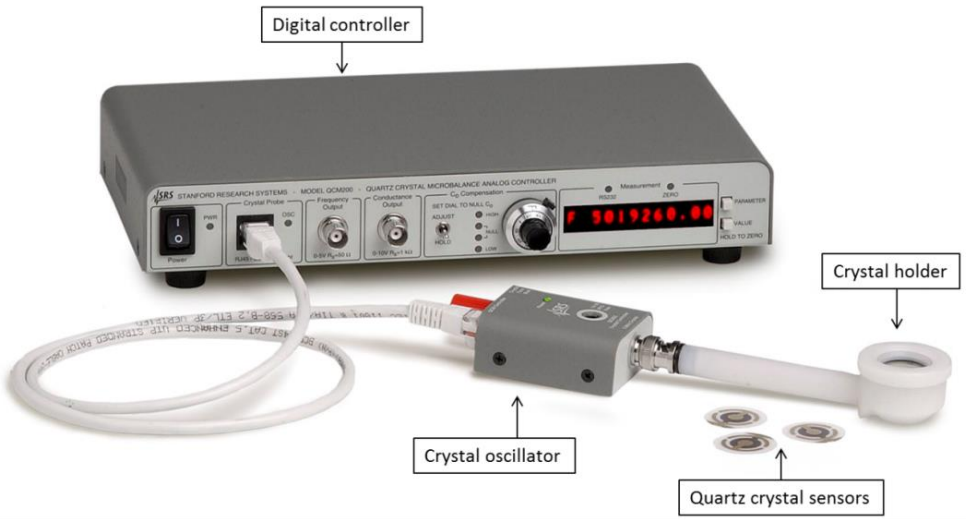

Figure 3-7: Complete QCM setup consisting of QCM200 Digital Controller, QCM25 Crystal Oscillator, Crystal Holder and quartz crystal sensor(s). Adapted from the device literature (*Stanford Research Systems, Inc.*, 2011, Revision 2.4).

3.5.2.2 Dose-dependent analysis

As this was the first time that volcanic ash has been administered at the ALI, an initial dose-dependent analysis of ash deposition, using feed masses to the dry powder insufflator of 4, 6 and 8 mg, was conducted to determine the dose that would be delivered to cells and the optimal dose to use for volcanic ash, specifically for the exposure experiments in ***Chapter 5***, which were chronologically performed first. The deposition was monitored using the QCM and indeed, showed a dose-dependent deposition of the ash sample (*Fig. 3-8*).

Insufflator load mass range (mg)	3-5	5-7	7-10
Average load mass (mg)	4	6	8
Average deposited mass (µg/cm^2)	0.21	0.45	0.89
Standard deviation	0.1	0.22	0.39
Standard Error of the Mean (SEM)	0.04	0.06	0.13
Number of experiments (n)	9	12	9

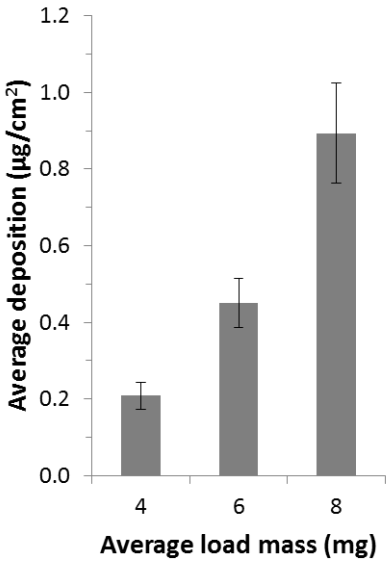

Figure 3-8: Dose-dependent analysis of volcanic ash deposition following nebulisation using a dry powder insufflator.

3.5.2.3 Dose-response analysis

Based on the dose-dependent analysis, an initial dose-response analysis of cytotoxicity, oxidative potential and (pro-)inflammatory response was performed (as described later in this *Section*) for the corresponding average dose (± standard error of the mean) that was deposited onto the cells of 0.13 ± 0.03 (n = 14), 0.21 ± 0.06 (n = 14) and 0.26 ± 0.09 µg/cm2 (n = 17) for 4, 6, and 8 mg starting (feed) mass, respectively.

Subsequent biochemical analysis indicated all doses to be sub-lethal to the co-culture system (*Fig. 3-9A*) with no change in cell morphology when compared

to the control sample (*i.e.*, untreated cells) (*Fig. 3-10*) nor clear dose-response in measured biochemical markers of oxidative stress and (pro-)inflammation (*Fig. 3-9B,C and D*). These findings suggested that the cell administered doses might have been too similar to be able to incite a significant and different response upon the cell cultures. Thus, it was decided to do a repeated exposure of the highest dose (8 mg) in order to increase particle mass deposition onto the cells and, hence, add an additional data point in the 'dose-response curve' (see ***Chapter 5***, *Section 5.2.2.1*). A mass of 8 mg was loaded three times into the dry powder insufflator and nebulised, aiming for a reliable difference in deposited dose to be used in comparison with the single exposure scenario (one nebulisation of 8 mg load mass).

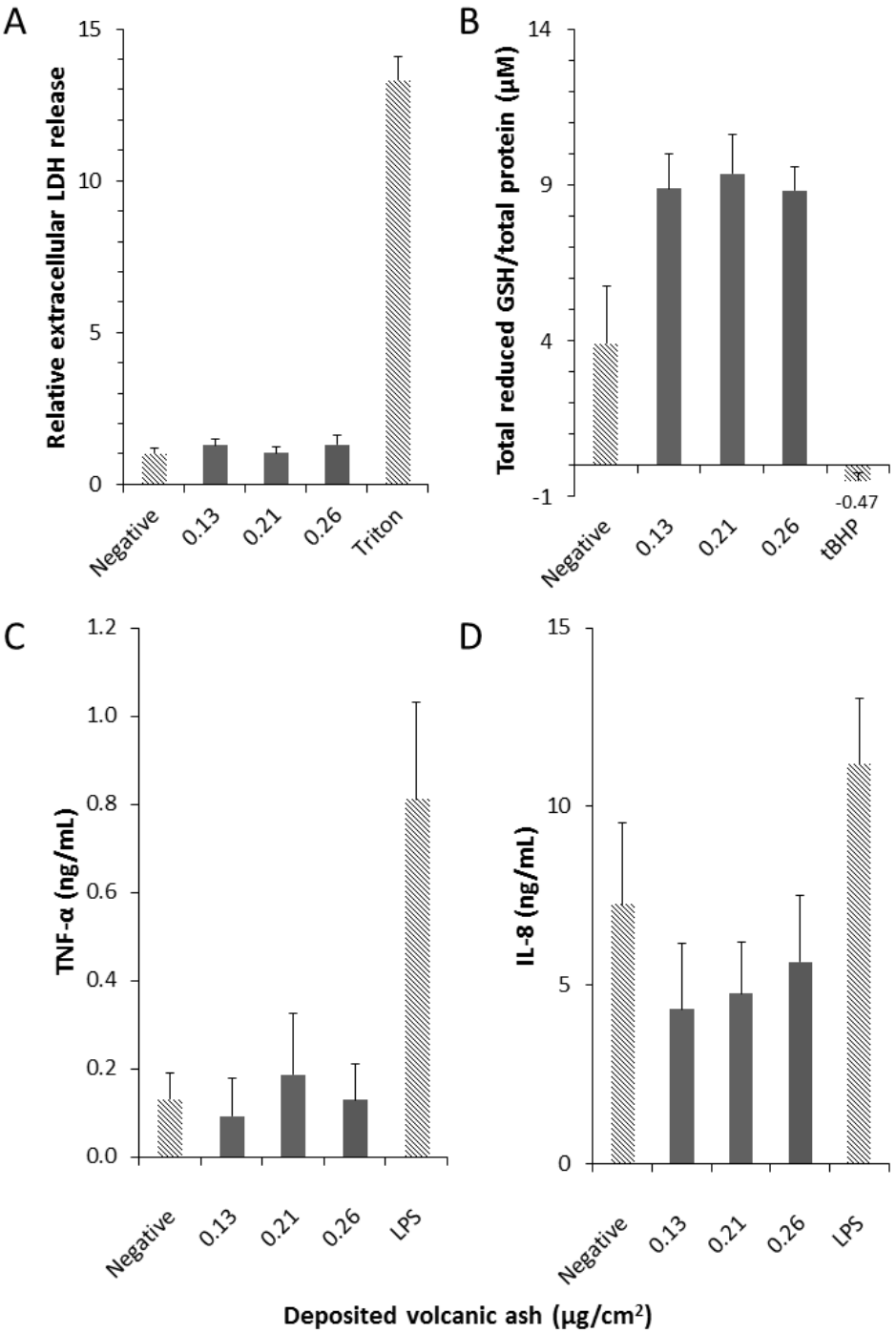

Figure 3-9: Initial biochemical dose-response analysis of the triple cell co-culture response following exposures to 0.13 µg/cm^2, 0.21 µg/cm^2, and 0.26 µg/cm^2 of respirable volcanic ash. **(A)** Cytotoxicity as determined by the release of lactate dehydrogenase (LDH), presented relative to the negative control (cell culture medium only) ± standard error of the mean. **(B)** Total reduced glutathione (GSH), **(C)** tumour necrosis factor-α (TNF-α), and **(D)** interleukin-8 (IL-8) release of the triple cell co-culture model after exposures, presented as the mean ± standard error of the mean. The respective positive assay controls are Triton X-100 (Triton; 100 µL of 0.2%), *tert*-Butyl Hydrogen Peroxide (*t*BHP; 250 µL of 100 mM) and lipopolysaccharide (LPS; 100 µL of 1 µg/mL).

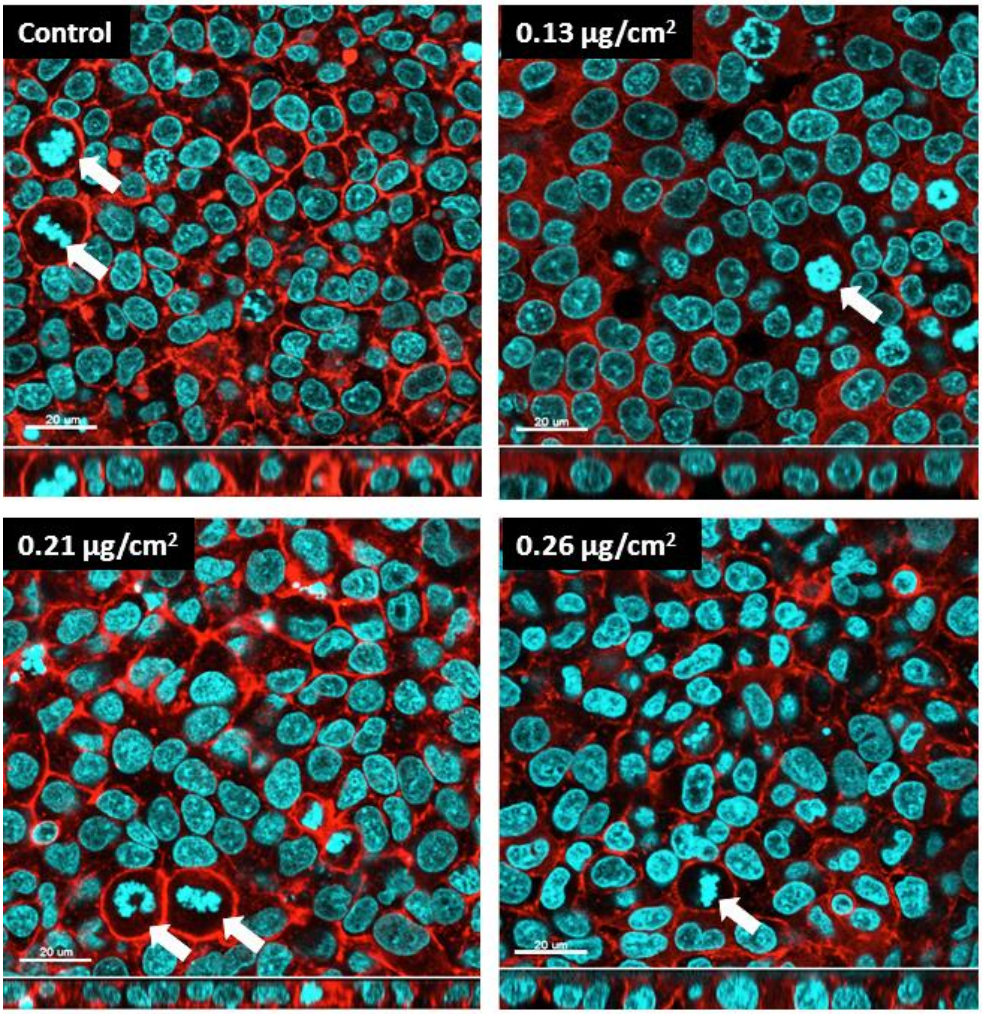

Figure 3-10: Cell morphology of the triple cell co-culture exposed to different exposure doses of volcanic ash. Confocal laser scanning microscopy (LSM) images show the F-actin cytoskeleton (red) and the nuclei (blue) of control and cultures exposed to respirable volcanic ash. White arrows indicate cells undergoing cell division. Scale bars are 20 µm. Images were collected at x63 magnification.

3.5.3 Cell imaging

To assess potential changes to the cell cultures *i.e.* alteration to cellular morphology following exposures, in comparison to the untreated cells, samples were visualised by laser scanning microscopy (LSM; *Section 3.5.3.1*). Interaction of ash with cells of the co-culture system was imaged using SEM, post-exposure (*Section 3.5.3.2*).

3.5.3.1 Confocal laser scanning microscopy

Following exposures and after the post-incubation period, the triple cell co-cultures were prepared for imaging via LSM. During collection, cell membranes were fixed

with 4% paraformaldehyde for 15 min at room temperature to preserve cells in their current state, and then washed and stored in phosphate buffered saline (PBS). Subsequently, they were permeabilised with 0.2% Triton X-100 in PBS for 15-30 min at room temperature, to enable the antibodies to cross the cellular membranes. Following the permeabilisation step, samples were blocked with bovine serum albumin (BSA) to minimize unspecific binding of the primary antibody within the cell. Samples were then stained with Phalloidin-Rhodamine (Molecular Probes, *Thermo Fisher Scientific Inc.*, USA) to label the F-actin cytoskeleton and 4',6-diamidin-2-phenylindole (DAPI; *Sigma-Aldrich*, Germany) to highlight the cell nuclei, diluted to 1:50 and 1:100 in 0.1% BSA in PBS for 1-2 h at room temperature, respectively. Then they were mounted with a mounting medium Glycergel (DAKO *Schweiz AG*, Switzerland) on microscope slides.

Visualisation of the samples was conducted with an inverted confocal LSM Zeiss 710 (*Carl Zeiss*, Switzerland) using a 63x/1.4 NA oil immersion lens. Representative images (z-stacks) were recorded at three independent fields of view for each sample and were further processed using the 3D reconstruction software IMARIS (Bitplane AG, Switzerland) or public domain image analysis software ImageJ (*http://rsb.info.nih.gov/ij*).

3.5.3.2 Scanning electron microscopy

Cell membranes were fixed with 4 % paraformaldehyde for 15 min at room temperature and then sequentially washed with 20, 40 and 60 % methanol for 5 minutes, 80 % methanol for 3 minutes and washed 5 times with 100 % methanol for 30 seconds. Samples were then dried in a vacuum desiccator over a 48 h period.

Subsequently, samples were carbon coated and imaged with a Mira3 LM (*Tescan*, Czech Republic) FE-SEM, using an InBeam detector on a rotated stage (60°) at the Adolphe Merkle Institute, University of Fribourg, Switzerland.

3.5.4 Cytotoxicity

The level of cellular cytotoxicity, *i.e.*, the potential of particles to cause cell membrane damage, was determined by measuring the activity of the intracellular

enzyme lactate dehydrogenase (LDH). LDH is a stable, cytoplasmic enzyme present in all cells, rapidly released into the cell culture supernatant upon damage to the plasma membrane.

The LDH activity was determined using an LDH cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany). In the first step of the assay (*Fig. 3-11*), NAD+ is reduced to NADH/H+ by the LDH-catalysed conversion of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H+ to the tetrazolium salt INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) which is reduced to formazan. The amount of LDH activity detected in the culture supernatant directly correlates to the amount of formazan formed during a limited time period and hence to the proportion of damaged and/or dead cells.

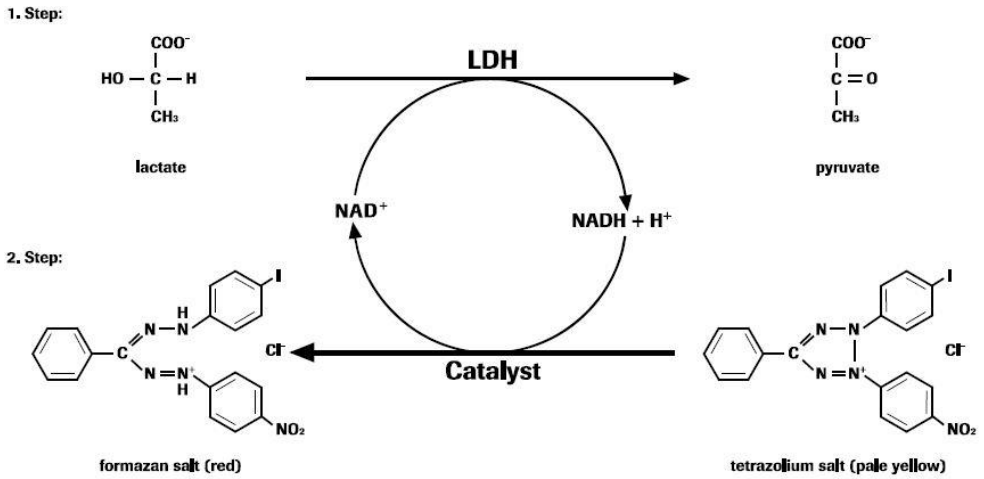

Figure 3-11: LDH detection principle. Figure from assay literature (*Roche Applied Science*, 2005).

Cell culture supernatants, collected post-exposure, were stored at +4 °C prior to analysis. The test was conducted on centrifuged, cell and particle-free supernatants. Supernatants were transferred in triplicate (each 100 µL) to 96-well plate and then incubated with the reaction mixture (diaphorase/NAD+ mixed with iodotetrazolium chloride and sodium lactate at a ratio of 1:45) from the kit, 100 µL per well. The absorbance was determined at 490 nm after 10 min using a microplate reader (*Bio-Rad*, Switzerland), with a reference wavelength set at 630 nm.

As a positive control, co-cultures were treated with 100 µL of 0.2 % Triton X-100 in H_2O on the apical side and incubated for 24 h at 37 °C, 5 % CO_2. The negative control was the supernatant from the untreated cells, *i.e.*, cell culture medium only.

3.5.5 Oxidative stress

The impact of any particle type upon the respiratory system is commonly associated with an increased level of oxidative stress (Donaldson et al., 2003) (***Chapter 2***, *Section 2.3.4.1*). Glutathione (GSH), a tripeptide (γ-gluthamylcysteinylglycine), is an important antioxidant widely distributed in plants and animals which is capable of preventing oxidative damage to cells by trapping reactive oxygen species such as free radicals, peroxides and metals (Pompella et al., 2003).

The total amount of intracellular reduced GSH in samples was quantified using a GSH assay kit (*Cayman Chemical Company*, USA). The assay utilizes an enzymatic recycling method, using glutathione reductase, for quantification of GSH (*Fig. 3-12*). The sulfhydryl group of GSH reacts with DTNB (5.5'-dithio-*bis*-2-(nitrobenzoic acid)) and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). Concomitantly, a mixed disulphide (GSTNB) is produced, which is then reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is, in turn, directly proportional to the concentration of GSH in the sample.

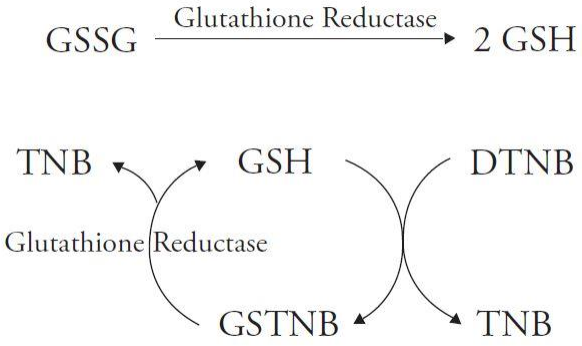

Figure 3-12: GSH recycling. Figure from assay literature (Assay Kit no. 703002; *Cayman Chemical Company*, USA, 2014).

Cells were scraped off from the insert membrane into the cell medium contained within the well and mixed with an equal volume of MES buffer (0.4M 2-(N-morpholino)ethanesulphonic acid, 0.1 M phosphate and 2 mM EDTA, pH 6.0; diluted 1:1 in H_2O). The entire volume of the well was then transferred to a 1.5 mL tube (A) and centrifuged at 4°C and 10,000 g for 15 min. Subsequently, ~200 µL of supernatant from tube A was transferred to tube C for assessing the protein levels in the sample (see *Section 3.5.5.1*3.5.5.1) and the rest into tube B for deproteination. As stated in the kit guidelines, almost all biological samples used for GSH measurement contain large amounts of proteins and it is necessary to remove as much protein as possible from the sample to avoid interferences due to particulates and sulfhydryl groups on proteins in the assay. In the first stage of deproteination, an equal amount of MPA reagent (metaphosphoric acid, dissolved 1:10 in H_2O) is added to the supernatant in tube B and mixed by vortexing. After standing at room temperature for 5 min, the sample was centrifuged at 4 °C and > 2,000 g for 5 min and the supernatant subsequently removed to the final set of tubes (D).

At this stage, samples can be stored at - 80 °C for later analysis; otherwise the following step is the second stage of deproteination, where 50 µL of TEAM reagent (4 M triethanolamine in H_2O) is added per mL of sample and vortexed immediately. 50 µL of the standard (provided in the kit, a stock of 25 µM GSSG in MES buffer) and deproteinated samples were added to each well in a 96-well plate in triplicate and mixed with 150 µL of the 'Assay Cocktail' which is prepared by mixing the following reagents: 11.25 mL MES buffer, 450 µL Co-factor Mixture (lyophilized powder of $NADP^+$ and glucose-6-phosphate; reconstituted with 0.5 mL of H_2O), 2.1 mL Enzyme Mixture (glutathione reductase and glucose-6-phosphate dehydrogenase in buffer; reconstituted with 2 mL of MES buffer), 2.3 mL water and 450 µL DTNB (5,5'-dithio-*bis*-(2-nitrobenzoic acid) as lyophilized powder; reconstituted with 0.5 mL of H_2O). The plate is then incubated in the dark on a shaker for 25 min, after which absorbance of samples is measured at 410 nm using a microplate reader (*Bio-Rad*, Switzerland).

GSH concentration in the samples is determined by the extrapolation of a standard curve. The results are expressed as total GSH content relative to the protein expression (*Section 3.5.5.1*) in the sample (GSH:protein; µM/mg) since it was not possible to gain information pertaining to the oxidative GSH component (GSSG) (Clift et al., 2014).

3.5.5.1 Protein concentration assay

The total protein content of the samples was determined by the Pierce BCA Protein Assay kit (Pierce Protein Research Products, *Thermo Scientific*, USA). This method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colourimetric detection of Cu^{+1} using a reagent containing bicinchoninic acid (BCA). The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. The absorbance of samples is nearly linear with increasing protein concentrations.

Samples from the test tubes C, prepared as described in *Section 3.5.5*, were diluted 10-fold with H_2O and then 25 µL of each sample and standards (provided in the kit, stock of bovine serum albumin (BSA) at 2.0 mg/mL in 0.9 % saline and 0.05 % sodium azide) were transferred to a 96-well plate in triplicate. The working reagent was prepared by mixing 50 parts of BCA Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of BCA Reagent B (4 % cupric sulphate). Using a multichannel pipette, 200 µL of the working reagent was added to each well and then mixed on a plate shaker for 30 s. After incubation at 37 °C for 30 min, the plate was cooled down to room temperature and the absorbance of samples measured at 562 nm using a microplate reader (*Bio-Rad*, Switzerland). The concentration of protein in each sample is determined based on the standard curve.

3.5.6 (Pro-)inflammatory response

Inflammation is considered to be a key process induced by respirable particles (*e.g.*, Stone et al., 2007). In response to interaction with particles, cells produce and

release mediators of (pro-)inflammatory response such as cytokines (***Chapter 2***, *Section 2.3.4.3*). These molecules have an important role in immune response, promoting the differentiation and division of immune cells and recruiting them to the site of inflammation (chemotaxis). In this way, cytokines are involved in various disease conditions such as chronic inflammation and cancer.

In this thesis, (pro-)inflammatory cellular response was investigated by quantifying tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8) and interleukin-1 β (IL-1β) release from the co-culture system *via* enzyme-linked immunosorbent assays (ELISA DuoSet Development Kit, *R&D Systems*) according to the manufacturer's protocol (*Fig. 3-13*).

A 96-well plate was coated with a cytokine-specific capture antibody 100 µL per well and incubated overnight at room temperature. The plate was washed 3 times with washing buffer (0.05 % Tween-20 in PBS) and then 300 µL of blocking buffer (1 % BSA in PBS) was added per well to block any non-specific binding sites. The plate was left for 1 h at room temperature and then washed prior to addition of 100 µL of sample and cytokine-specific standards in triplicate, resulting in binding of any target cytokine present in the sample to the capture antibody. After 2 h incubation at room temperature and subsequent washing, 100 µL of suitable detection antibody was added to each well to bind the target cytokine, which was followed by a further 2 h incubation. After washing, 100 µL of streptavidin conjugated to horseradish peroxidase (HRP) enzyme was added to each well for 20 min. Streptavidin binds to biotin, which is associated with the secondary antibody and is bound to any detected cytokine present. After washing, 100 µL of the substrate solution (1:1 Colour reagent A – H_2O_2 and Colour Reagent B – tetramethylbenzidine) was added per well for 20 min, leading to a colourimetric reaction which converts HRP into a detectable signal (blue colour). To stop the reaction, 50 µL of 1 M H_2SO_4 was added per well which resulted in a yellow colour. The plate was mixed on a plate shaker for 2 min and then the absorbance of samples was read at 450 nm using a microplate reader (*Bio-Rad*, Switzerland).

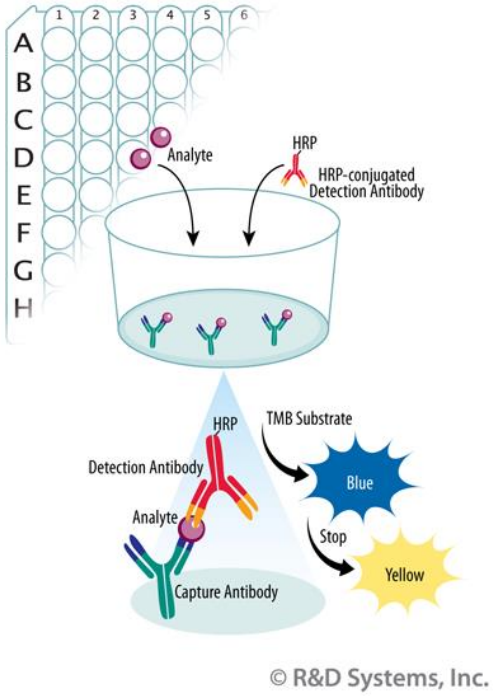

Figure 3-13: Scheme of sandwich ELISA. Taken from *R&D Systems, Inc.* website (*www.rndsystems.com*).

Sample cytokine concentrations were determined via extrapolation of a recombinant cytokine protein standard curve. Lipopolysaccharide (LPS, from *E. Coli* at 1 µg/mL) was applied as 100 µL solution in the bottom compartment of the co-cultures and served as the positive control for TNF-α, IL-8 and IL-1β induction. The negative control was the cell culture medium only.

3.5.7 Gene expression analysis

One of the results of particle exposures may be upregulation of, *e.g.,* oxidative stress or (pro-)inflammatory marker gene expression in cells; hence, for the studies presented in this thesis, gene expression was assessed as follows. The induction of cell death was determined by expression levels of (pro-)apoptotic genes caspase 7 (*CASP7*) and FAS receptor (*FAS*). Heme oxygenase 1 (*HMOX1*) and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*) genes were used to observe the onset of oxidative stress. For assessment of (pro-)inflammatory responses, expression of interleukin-1 beta (*IL1B)* and interleukin-8 (*IL8*) genes were measured.

Quantification of gene expression at the transcriptional level was performed by a commonly used, sensitive method – a reverse-transcriptase polymerase chain reaction (RT-PCR) (*Fig. 3-14*). The method requires the reverse transcription (RT) of ribonucleic acid (RNA) into complementary deoxyribonucleic acid (cDNA), which is followed by PCR amplification of the cDNA (*i.e.*, production of copies of the target DNA). The level of RNA transcripts (cDNA) is calculated from the number of the PCR cycle determined by the detection of fluorescence signals that exceed a certain threshold.

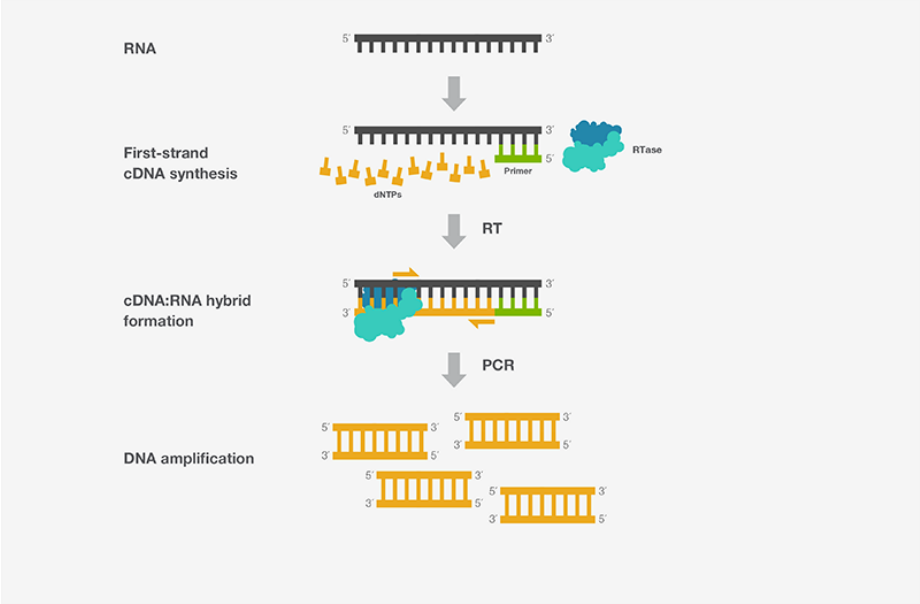

Figure 3-14: Reverse transcription polymerase chain reaction (RT-PCR). Taken from ThermoFisher Scientific website (*www.thermofisher.com*)

The analysis procedure is briefly outlined here; for a detailed protocol, the reader is referred to the respective handbooks of the assay kits employed (see below).

Cell culture membranes, sampled following particle exposures, were stored in the RNA protect buffer (RNAprotect® Cell Reagent, *Qiagen,* Germany) prior to the analysis. Before RNA isolation, the membrane was vortexed to detach the cells into the buffer. RNA was isolated with RNeasy® Plus kit (*Qiagen,* Germany), according to the protocol in *RNeasy Plus Universal Handbook* (version 12/2014; p. 16-21; *www.qiagen.com*). In the procedure, the cells were lysed and homogenised, and

then the homogenate was separated into aqueous and organic phases. RNA was collected and purified from the aqueous phase and subsequently, the yielded total concentrations RNA of samples were analysed with a NanoDrop micro-volume spectrophotometer (*Thermo Scientific*, USA).

The RNA-aliquots were then used to synthesize the cDNA. cDNA synthesis *i.e.*, RT reaction was done using the Omniscript® RT kit (*Qiagen,* Germany), according to the protocol in the *Omniscript Reverse Transcription Handbook* (version 10/2010; p. 11-14; *www.qiagen.com*). To start, the reaction requires a reverse transcriptase (RTase) enzyme and primers, which bind to RNA sequences on the sample, to which the enzyme then binds to, thus initiating the cDNA synthesis. Here, we used Oligo-dT primer (*Microsynth,* Switzerland). Other components necessary for an efficient RT reaction include a buffer, dNTP, DTT, RNase inhibitor (*Promega,* USA) and RNase-free water.

Then, the portion of the resulting cDNA with the addition of gene-specific primers was amplified by PCR. A real-time PCR was performed using SYBR® Green (a reagent incorporating fluorophores which generate fluorescence signals; *Applied Biosystems,* USA) on a 7500 Fast Real-Time PCR system (*Applied Biosystems,* USA).

Relative gene expression levels were calculated using the $\Delta\Delta C_t$ method (*e.g.*, Livak and Schmittgen, 2001), with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the standard (housekeeping) gene.

3.6 Data processing and statistical analysis

Statistical analyses were performed either using statistical software SPSS (*IBM* SPSS Statistics for Windows, Version 22.0, USA) or Origin (version 9.3, *OriginLab Corporation*, USA). Statistical significance was deduced through the use of a one-way analysis of variance (ANOVA), assuming a normal distribution of the datasets. Subsequent Tukey's post hoc tests were conducted to determine statistical

significance between different exposures and the reference exposure. The alpha value was set at 0.05.

3.7 References

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Part II

The plume environment and its impact on the respiratory toxicity of volcanic ash

Chapter 4

An *in vitro* study of the potential respiratory hazard of plume-exposed volcanic ash

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4.1 Introduction

Volcanic plumes are complex environments composed of gases, aerosols and ash, where various chemical and physical reactions occur over a spectrum of temperatures (ranging from magmatic to stratospheric) and timescales (seconds to days). By processes of chemical reaction and adsorption onto the surface of ash particles, volatiles (such as sulphur and halogen gases and metals) can be scavenged, *i.e.*, removed from the atmosphere, commonly in the form of soluble and insoluble salts adhered to ash surfaces (see ***Chapter 2***, *Section 2.6*). These generally consist of various sulphates and halides, including $CaSO_4$, Na_2SO_4, NaCl, NaF, $CaCl_2$, CaF_2, K_2SiF_6, Na_2SiF_6 and AlF_3 (Cronin et al., 2003, de Moor et al., 2005, Delmelle et al., 2007, Gislason et al., 2011). The presence and nature of the salts is commonly investigated by leachate studies (Witham et al., 2005, Stewart et al., 2013), which have shown that the most abundant readily-soluble elements typically observed in volcanic ash leachates (extracted by water) are Ca^{2+}, Na^{+}, Mg^{2+}, SO_4^{2-} and Cl^{-} (Ayris and Delmelle, 2012).

The potential impact of the presence of salt coatings on the respiratory toxicity of volcanic ash is unknown. In general, leachable components on particle surfaces, such as trace metals, can have an important role in driving adverse respiratory health effects following exposure to ambient particles (*e.g.*, Gilmour et al., 1996, Carter et al., 1997, Fubini et al., 1995b, Rice et al., 2001, Fubini, 1997). Particles containing chelatable metals are known for their potential to induce oxidative stress *via* free radical generation upon contact with lung structures, and have also been found to stimulate the release of (pro-)inflammatory markers (Cho et al., 2012, 2010, Smith et al., 2000). Furthermore, various studies have associated exposure to airborne sulphate particles with changes in pulmonary function and particle clearance rates (Chen et al., 1992, Green et al., 1995, Koenig et al., 1983, Lippmann and Thurston, 1996), and enhanced respiratory symptoms, such as chronic cough and bronchitis, specifically in children and other susceptible individuals (Dockery et al., 1996, Gwynn et al., 2000 and references therein). These effects were mainly attributed to the elevated levels of particle acidity (H^{+}), to

which sulphate (SO_4^{2-}) concentration has traditionally been associated (Hazi et al., 2003, Gwynn et al., 2000, Lippmann, 1989). For volcanic ash, it has been postulated that surface species, if leached from the particle surface, could be a potential cause, at least in part, of reported irritancy to the eyes, throat and airways following exposure to freshly erupted ash (Horwell and Baxter, 2006, Searl et al., 2002, Witham et al., 2005, Hansell et al., 2006).

The aim of the present study was to investigate, for the first time, the biological impact of in-plume volatile scavenging on ash toxicity. To achieve this, a multicellular *in vitro* human lung model was exposed at the air-liquid interface (ALI) (see ***Chapter 3***, *Section 3.5.1*) to simulated respirable ash (as real ash is already plume-exposed) which was either salt-laden (produced through simulated high-temperature in-plume processing, as explained below) or pristine.

4.2 Methods

To emplace surface salts via replication of chemical reactions that occur between pristine ash surfaces and volcanic gas during an eruption, the Advanced-Gas and Ash Reactor (AGAR), a sophisticated experimental setup capable of simulating conditions of an eruptive plume, was used (Ayris et al., 2015) (as described in *Section 4.2.2*). The experiments were conducted using analogue substrates, *i.e.*, pulverised synthetic volcanic glass and natural pumice (*Section 4.2.1*), in order to obtain fresh particle surfaces (which had not previously been exposed to gases in a volcanic plume). The glass represents a homogenous proxy particle for the primary constituent of volcanic ash which allows assessment of the impact of surface changes without the additional effects of the natural substrate heterogeneity. It is also the primary phase contributing to diffusion-driven salt formation (Sucov and Gorman, 1965, Ayris et al., 2013, 2014), thereby ensuring maximum salt loading of the samples for *in vitro* toxicity assessment. Crushed pumice was additionally used because it is representative of a real, heterogeneous volcanic substrate, as was also done by Damby et al. (2018). Samples were exposed to a single gas, sulphur dioxide

(SO_2), at a single temperature (500 °C), thus generating salt-laden particles in controlled conditions (as described in *Section 4.2.3*).

Initially, the experiments were designed to include particle exposures to HCl gas and a mixed SO_2-HCl atmosphere, in addition to SO_2 gas, in order to understand the effects of different types of salt coatings (sulphate versus halide) and their combination on ash toxicity. To keep the experimental conditions consistent throughout the exposure experiments with different gases, as well as to ensure successful formation of salt coatings on the samples, a single temperature of 500 °C was chosen. In response to high temperature exposures, the bonding environment of the surface and bulk structure of the glass may be altered, and thus, the number and reactivities of surface sites for chemisorption of gases (see Ayris, 2010). It has been shown in the past that the optimal efficiency of HCl uptake under experimental conditions in AGAR occurs up to 500 °C (Ayris et al., 2014), whereas SO_2 uptake increases exponentially with temperature up to 600 °C, after which it reaches a plateau (Ayris et al., 2013). However, when the glass sample that had been exposed to HCl (as described for SO_2 in *Section 4.2.3*) was separated to retrieve the near-respirable fraction for toxicological analysis (***Chapter 3***, *Section 3.3.1*), it was observed that the material recovery was negligible. Visualisation of the sample by SEM showed aggregation of particles (***Appendix 1***), which most likely occurred during the experiment via cementation of salts between particles. Due to their hygroscopic nature, halite salts may enhance the uptake of H_2O (Gilbert and Lane, 1994), which can create a liquid film on the particle surface and, thus, result in formation of aggregates (*e.g.*, Mueller et al., 2017, Van Eaton et al., 2012). Since the mixed SO_2-HCl sample would have both sulphates and halides as reaction products, it was assumed this sample would also be affected by these phenomena, and thus, this sample was not generated. Since breaking up the aggregates to improve recovery would mean secondary processing (and potential changes to the particle surface), it was decided not to proceed with conducting toxicology experiments using HCl and SO_2-HCl treated samples. Data for the exploratory analyses of HCl and SO_2-HCl-treated samples can be found in ***Appendix 1***.

As was done in ash toxicity testing experiments throughout the thesis, near-respirable (sub-4 µm) fractions of AGAR-generated samples were isolated from the bulk samples (as described in ***Chapter 3***, *Section 3.3.1*) for subsequent use in cell-based toxicity assessments (as described in *Section 4.2.6*). The respirable samples were characterised for their particle size, imaged to confirm the presence of surface salt coatings and their bio-solubility was measured using techniques described in ***Chapter 3*** (*Section 3.4*), except where sample quantities were insufficient.

4.2.1 Volcanic glass and pumice samples

As mentioned in *Section 4.2*, for the *in vitro* toxicity assessment, rather than using a range of natural samples that likely have an unconstrained salt load due to plume exposures, analogue materials were used instead. These were powdered volcanic glass and pumice samples (compositionally equivalent to the Soufrière Hills volcano (SHV) ash used in other studies in the thesis), as proxies for volcanic ash, with salt deposits emplaced on their surface in constrained experiments using the AGAR (*Section 4.2.3*).

A glass powder of andesitic composition was synthetized by melting a sample of pumice rock sourced from the Soufrière Hills volcano, Monserrat (from the 12 July 2003 eruption) at 1300 to 1600°C. The melt was homogenized and then rapidly quenched to produce glass, which was ground to a powder using an agate planetary ball mill. The 'natural ash' material was generated by hand crushing the pumice to a powder using an agate pestle and mortar.

4.2.2 Advanced Gas-Ash Reactor

The Advanced Gas-Ash Reactor (AGAR) is an instrument that allows for experimental investigation of gas-ash interactions which may occur during a volcanic eruption (Ayris et al., 2015). In this study, it was used to simulate SO_2 uptake by volcanic glass and ash (*i.e.*, crushed pumice) samples at temperatures relative to eruptive column temperatures (see ***Chapter 2***, *Fig. 2-3*), in order to emplace salt deposits at the particle surface. This approach was based on previous experiments, where silicate glasses with a range of representative volcanic

compositions were exposed to SO_2 (Ayris et al., 2013). These experiments demonstrated that the uptake of SO_2 at temperatures above 300°C occurs via rapid adsorption onto reactive surface sites followed by the formation of anhydrite ($CaSO_4$) deposits at the particle surface (see ***Chapter 2***, *Section 2.6*). This is the first time the instrument had been used in order to investigate the potential biological impacts of such gas-ash interaction.

The instrument (*Fig. 4-1*) is composed of a horizontal tube furnace with three heating stages separated by thermal barriers, allowing operation at variable heating rates and different hold temperatures. The furnace (*Fig. 4-1A*) is loaded with a fused quartz working tube which can be rotated, enabling tumbling of material during the experiments for maximum surface-gas interaction. A gas injector assembly (*Fig. 4-1B*) is fitted at the tube inlet that enables aerosolization of deionised water by a flow of argon gas and delivery of an additional gas stream around the nebuliser into the working tube.

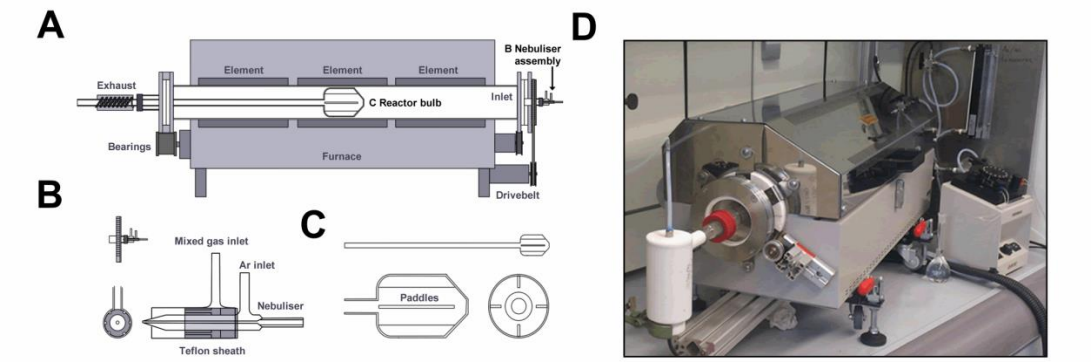

Figure 4-1: Schematic of the Advanced Gas-Ash Reactor (AGAR), displaying **A)** the furnace, **B)** gas injector, **C)** sample bulb and **D)** a photograph of the device *in situ*. Produced by Paul M. Ayris (Ayris et al., 2015).

The sample is loaded into a quartz sample bulb (*Fig. 4-1C*), capable of holding up to 10 g of particulate material, which contains paddles to ensure sample mixing during rotation. The bulb is then inserted into the working tube and placed in the stage of the furnace with the desired temperature. The exhaust gas is vented *via* an outlet at the end of the sample bulb to an extractor system and within-laboratory fumehood.

The details of the instrument design and operational conditions are described in Ayris et al. (2015).

4.2.3 Salt-laden particle generation

Around 6 g of sample per run (*Section 4.2.2*) was loaded into the quartz sample bulb and placed within the horizontal tube furnace and heated to a temperature of 500°C. All experiments were carried out under a 25 SCCM (Standard Cubic Centimeters per Minute) gas stream of SO_2 and 75 SCCM of argon (Ar) carrier gas, exposing glass and pumice ash samples for a total of 120 min. Control samples for both materials were produced under a 100 SCCM Ar flow only for 120 min.

After the experiment, samples were removed from the reactor and left to cool to ambient temperature, and then stored in sealed vials prior to the isolation of the respirable fraction. Experiments were conducted on three different occasions for each (final) particle type (*Fig. 4-2*) and then combined in order to maximise the amount of material available for the study.

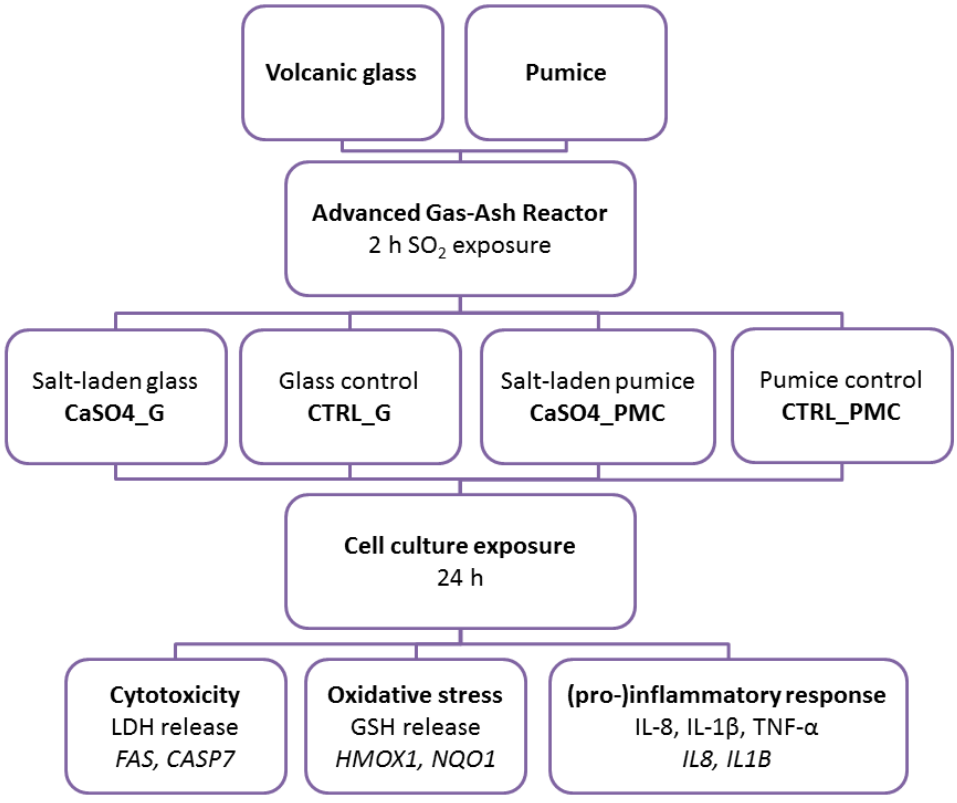

Figure 4-2: Schematic to demonstrate how artificially generated ash and volcanic glass samples were exposed to SO_2 for 2 h in the Advanced Gas-Ash Reactor and used in toxicology assessment.

4.2.4 Physical characterisation of particles

The particle size distribution of isolated respirable post-AGAR samples were determined using a Coulter LS230 laser diffractometer (*Coulter Corporation*, USA) in water without sonication, at the Ludwig-Maximilian-University Munich, Germany. Results are the mean of three consecutive runs of each sample.

Imaging of particle morphology and surface textures was carried out on a JEOL JSM-6500F field emission scanning electron microscope (FE-SEM; *JEOL*, USA) at the HP-HT Laboratory of INGV Rome, Italy.

4.2.5 Biosolubility of surface salt deposits

The concentrations of readily water-soluble surface elements (*i.e.*, salt coating) from artificially generated ash were measured. This was done in order to identify the species adsorbed onto the surfaces of particles during the experiments in AGAR. In addition, to estimate if particle coatings were likely to still be on the surface at the point of cellular uptake, so that any toxicological outcomes could be better understood, the rate of dissolution was assessed. For this purpose, samples would ideally be leached in a simulated lung fluid (SLF), to resemble the environment particles would encounter following deposition in the lungs. However, instead, a water leach was used due to the insurmountable complexities encountered during the SLF method development (described in ***Chapter 3***, *Section 3.4.5*). It was shown that a water leach can be used as a good indicator of species that will dissolve in a saline solution (such as Gamble's solution), since the results among different proxies for SLF were found to be similar.

Samples were leached in water as described in ***Chapter 3***, without agitation. Briefly, 0.2 g of the post-AGAR (bulk) samples were leached with deionised water at a ratio of 1:100 (g dry weight ash to mL water) in three replicates, each with a contact time of 1 hour at room temperature in static conditions. Additional tests were performed with contact times of 10 min and for 30 min (one replicate each) to assess the rate of dissolution of particle coatings.

Subsequently, subsamples of the leachates were filtered through 0.2 µm and 0.45 µm filters for anion and cation analysis, respectively. The concentrations of major cations (Ca, Mg, Na, K) and trace elements (Al, Fe, Mn, Zn, Cu) in collected extracts were measured by inductively coupled plasma-mass spectrometry (ICP-MS). Anion analysis (Cl^-, F^-, SO_4^{2-}, PO_4^{3-}, NO_3^-) of water leachates was carried out using ion chromatography (IC). The results were expressed in mg element per kg of dry sample weight.

4.2.5.1 Solubility modelling

To investigate which species might dissolve (or could potentially precipitate) when in contact with SLF (*i.e.*, upon contact with the cell layer surfactant in *in vitro* experiments) at 37 °C at pH 7.4, geochemical modelling using PHREEQC software for Windows (Parkhurst and Appelo, 2013) was conducted. Simulations were performed by creating an aqueous solution with the given concentrations of different salts used to make the SLF (Gamble's solution; Moss, 1979) (*Table 3-1* in ***Chapter 3***, *Section 3.4.5*). The solution was equilibrated to atmospheric CO_2 and pH adjusted to 7.4. This solution was modelled to determine the concentrations and activities of all the possible species present. In addition, the saturation index for every possible precipitating phase was calculated. In a second step, the measured water-leach concentrations for post-AGAR samples were added to the SLF solution (as $CaSO_4$, Na_2SO_4 and $MgSO_4$ salts). The objective of these simulations was to infer what would happen to the solution (*i.e.*, if any phase will become supersaturated) if similar amounts of sulphate salts were dissolved.

A separate batch of simulations were performed to investigate the speciation of the water-leach experiments at the different times investigated (10, 30 and 60 min). In this case, a pure water solution was equilibrated with CO_2 until a pH of 6 (similar to the pH value normally measured on laboratory deionized water) was achieved, then, $CaSO_4$, Na_2SO_4 and $MgSO_4$ salts were dissolved to achieve the same cation concentrations as those measured at the different experimental times.

4.2.6 Cell culture exposures

The multicellular lung model at the ALI was separately exposed to respirable salt-laden and control glass and pumice samples (*Fig. 4-2*) in order to deduce the response to either salt-laden particles or the particle substrate (control sample) alone.

Whilst testing the samples, using the approach where 'fixed' sample feed masses were loaded into the dry powder insufflator (as described in ***Chapter 3***, *Section 3.5.2.1*), it became apparent that achieving an equivalent mass exposure and reproducibility for different samples was challenging. The variations in deposition arose mainly due to the handling of the equipment which is also influenced by the nature of the chosen particles (*e.g.*, the internal surfaces of the device may be coated with powder, hence, influencing the powder discharge). In the attempt to overcome the issues, an approach was applied where the deposition was monitored during the exposures (using the QCM) and the ash was nebulized until a targeted dose between 0.5 and 0.7 µg/cm^2 was reached, regardless of the feed mass. The doses used, however, likely deviate from a real-life exposure and represent an over-load scenario (see discussion in ***Chapter 5***).

After a 24 h post-exposure incubation period, cell cultures were assessed for biological endpoints relevant for common particle-induced toxicity mechanisms (described in ***Chapter 2***, *Section 2.3.4*), including cytotoxicity (LDH release), oxidative stress (depletion of intracellular GSH and oxidative stress-related gene expression) and (pro-)inflammatory response (TNF-α, IL-8 and IL-1β at gene and protein levels). The impact of particle exposures upon cell morphology, as well as interaction with the multicellular model, was visualised *via* confocal laser scanning microscopy (LSM) and scanning electron microscopy (SEM), respectively. Additional information about the bioanalysis assays performed is outlined in ***Chapter 3***, *Section 3.5*.

4.3 Results

4.3.1 Physical characterisation of particles

Results of the particle size analysis of isolated respirable fractions showed the proportion of particles sub-10 µm and sub-4 µm in diameter in samples was 93 ± 4.3 and 45.1 ± 1.6 vol. %, respectively (*Table 4-1*; *Fig. 4-3*).

Table 4-1: Particle size results for post-AGAR samples.

Sample	Particle size (cum. vol. %)			
	< 1 µm	**< 2.5 µm**	**< 4 µm**	**< 10 µm**
CaSO4_G	13.2	25.9	45.2	95.6
CTRL_G	10.8	26.6	47.3	97.7
CaSO4_PMC	7.0	22.8	43.7	90.0
CTRL_PMC	5.9	23.4	44.1	88.6

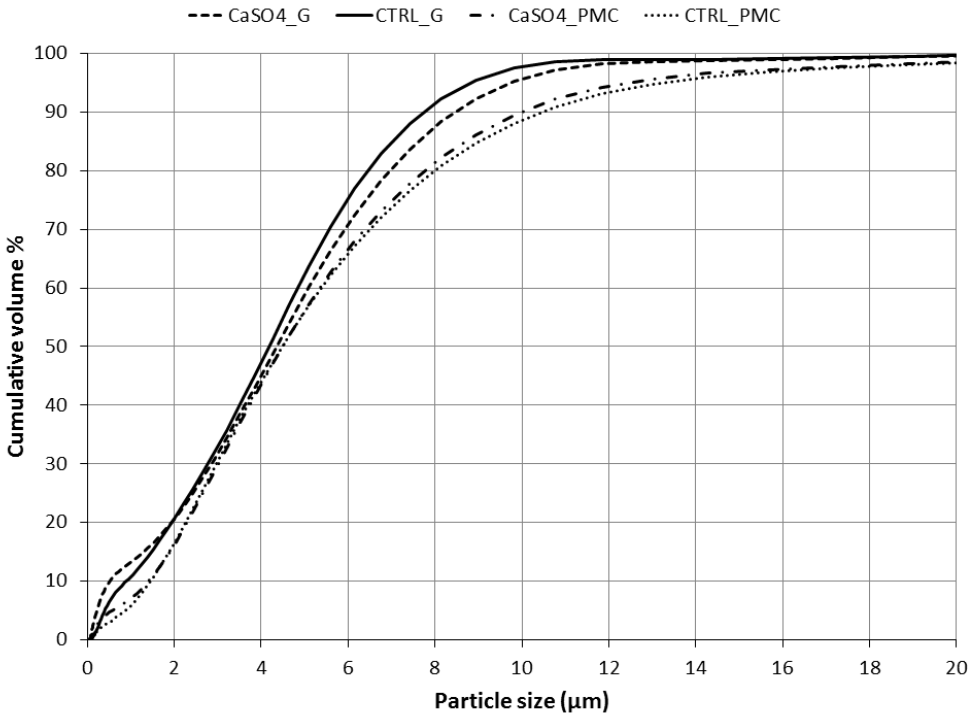

Figure 4-3: Particle size distribution of respirable post-AGAR samples. Three measurement cycles were recorded for each sample and an average taken.

The generated particles were shown to be good analogue materials for volcanic ash with regards to their shape, as observed by SEM. Similar to the morphology generally observed for respirable volcanic ash (*e.g.*, Damby et al., 2016, Lahde et al., 2013, Horwell et al., 2013, Hillman et al., 2012, Le Blond et al., 2010), the particles were mostly blocky and angular with varying amounts of sub-micron particles adhering to the surfaces of larger particles (*Fig. 4-4*). The SEM analysis of generated ash particles also revealed an abundance of discrete salt deposits in the form of sub-micron sized, nodule-like crystal structures across the particle surfaces (*Fig. 4-5*).

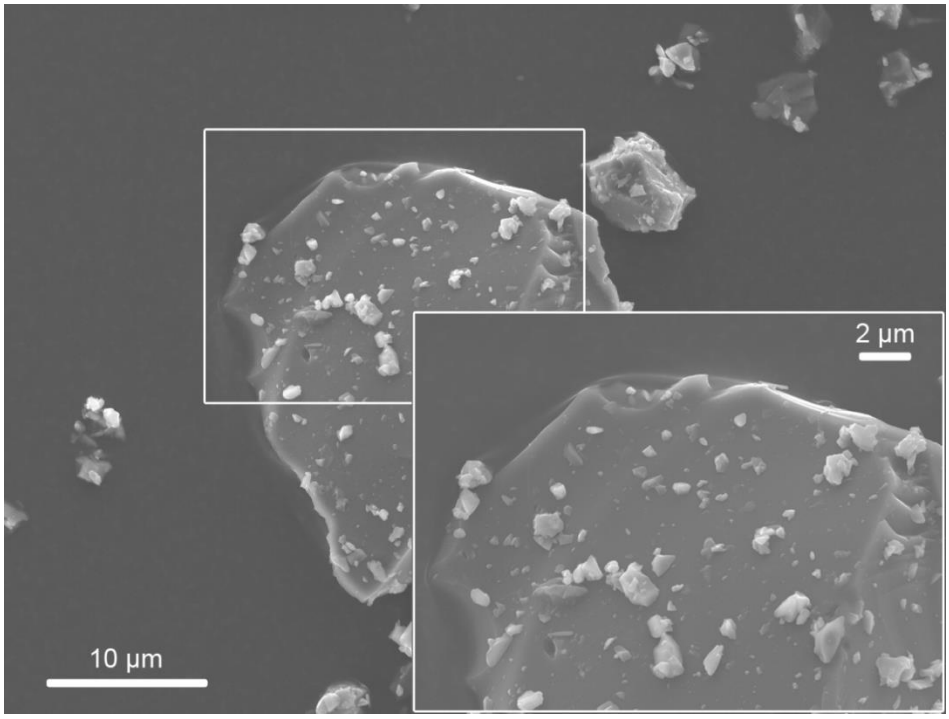

Figure 4-4: Representative scanning electron micrographs of synthetic volcanic glass particles exposed to Ar (CTRL_G) for 2 h in the Advanced Gas-Ash Reactor (AGAR). Images were collected at 10.0 kV and 8.1 mm working distance. Scale bars are noted on the images.

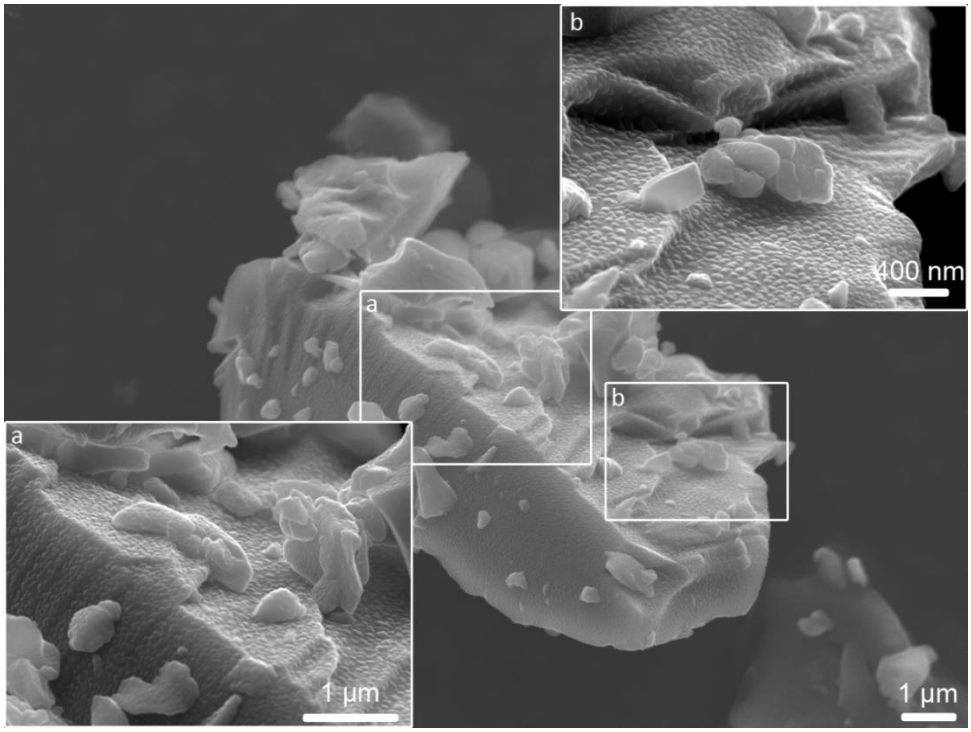

Figure 4-5: Representative scanning electron micrographs of salt-laden synthetic volcanic glass particles exposed to SO_2 ($CaSO_4$_G) for 2 h in the Advanced Gas-Ash Reactor (AGAR). The result of interaction is formation of $CaSO_4$ on the particle surface, in the form of surface nodules, as shown in insets **a)** and **b)**. Images were collected at 10.0 kV and 9.1 mm working distance. Scale bars are noted on the images.

4.3.2 Biosolubility of surface salt deposits

The presence and the nature of the surficial salt deposits on the post-AGAR samples are indicated by the leachate data (60 min), where the dominant species in the water leachate solutions were Ca^{2+} and SO_4^{2-} (*Fig. 4-6*, *Table 4-2*). This is the case as well when compared to a water leach of SHV natural ash used in this thesis (see ***Chapter 3***, *Section 3.2.1*), with the exception of soluble Na^+.

The time-series experiment showed that dissolution of species is relatively rapid, with the majority of available soluble species (as measured after 60 min) coming off the particle surface within the first 10 min of leaching (*Fig. 4-7*).

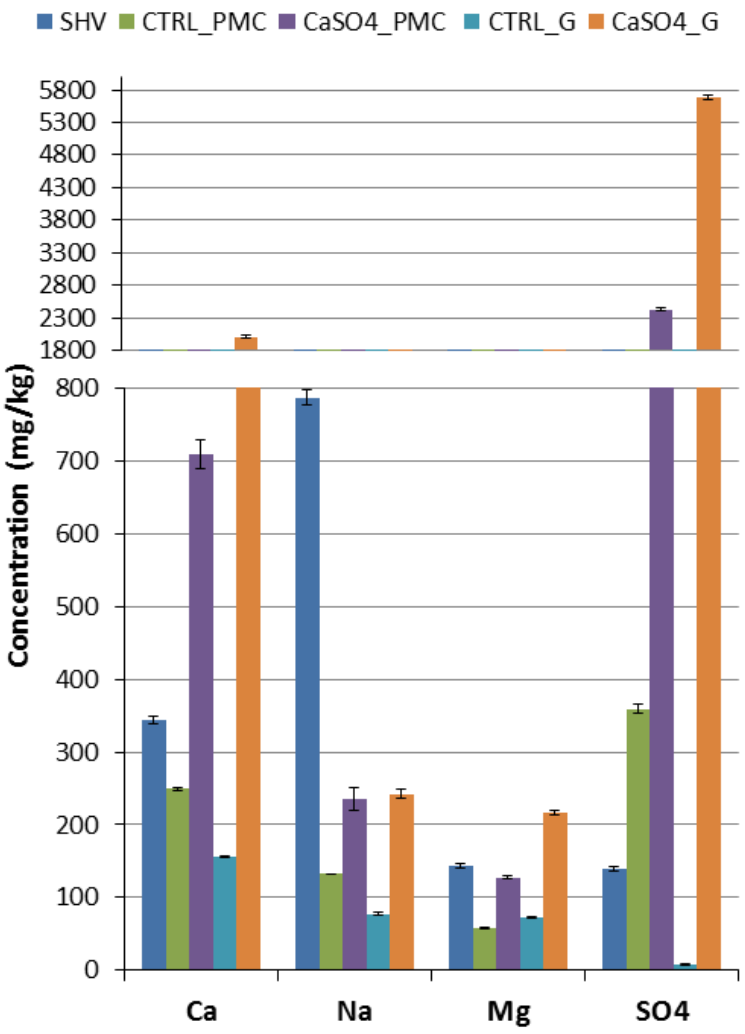

Figure 4-6: Comparison of soluble element concentrations (mg/kg) in the post-AGAR samples and natural SHV ash measured after 1 h of leaching in deionized water. Data are shown as mean concentration ± standard error of the mean (n=3).

Table 4-2: Mean concentration (± standard error of the mean; mg/kg) of water-extractable major elements from the samples used in the present study and from the global dataset (Ayris and Delmelle, 2012)*.

	Ca	S	Na	Mg
CaSO4_G	2008 ± 12.7	1893 ± 11.9	242 ± 6.3	217 ± 3.2
CTRL_G	155 ± 1.2	3 ± 0.3	78 ± 1.4	72 ± 1.1
CaSO4_PMC	710 ± 19.9	806 ± 7.5	236 ± 16.3	128 ± 1.8
CTRL_PMC	249 ± 2.2	120 ± 2.2	132 ± 0.4	58 ± 0.7
Global dataset*	2172	1711	407	349

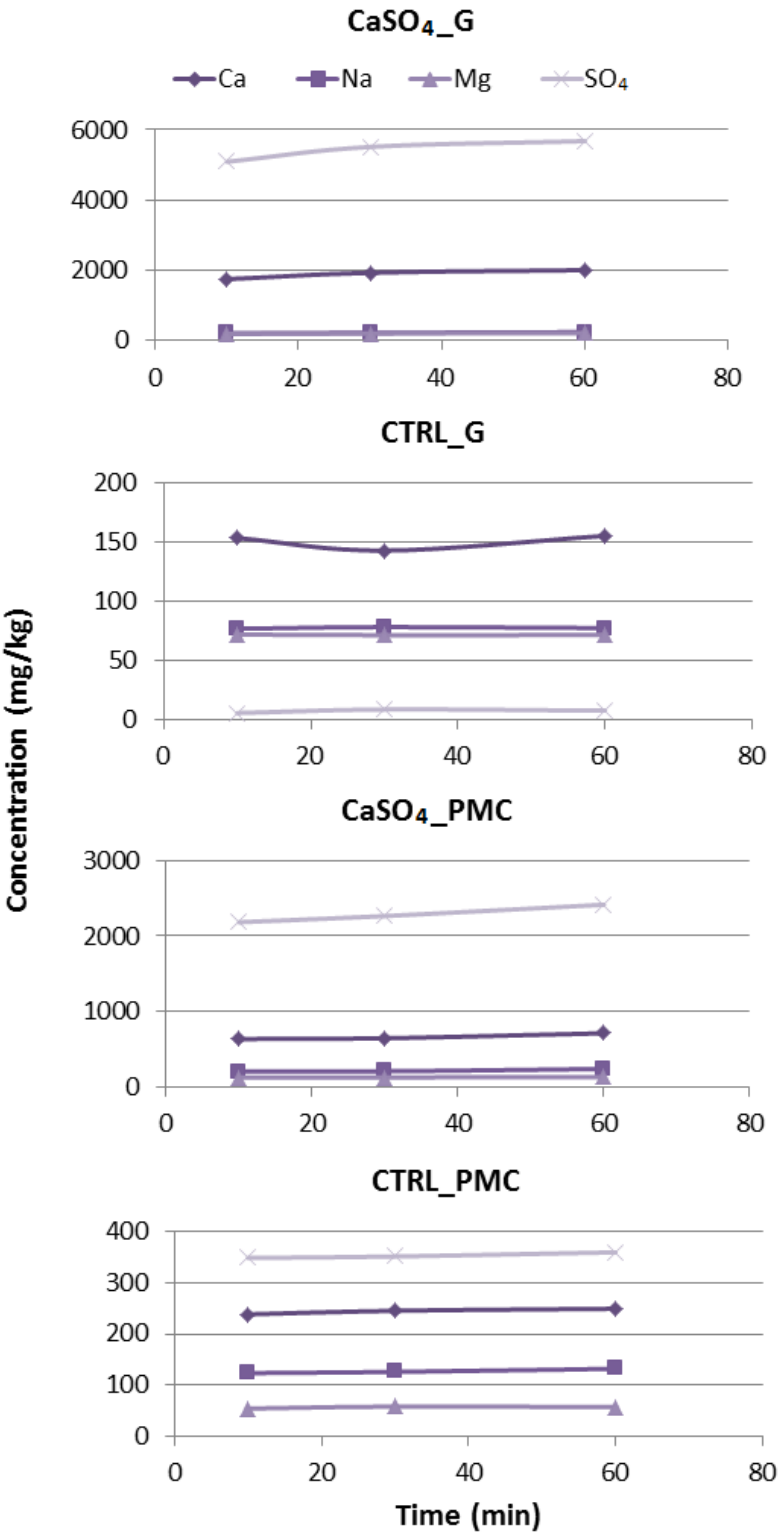

Figure 4-7: Plot of element concentration (mg/kg) in glass and pumice samples after 2 h SO_2 experiments in solutions extracted after 10, 30 and 60 min leaching in deionized water. The data for 60 min time-point are the same as shown in Figure 4-6 (*i.e.*, mean concentration of n=3), while extractions after 10 and 30 min were done in one replicate only (n=1).

PHREEQC modelling of the water leachate solutions showed that, at the three experimental times (10, 30 and 60 min), the solutions were well undersaturated with respect to $CaSO_4$ (*Fig. 4-8*), with saturation index values ranging from -2.53 (10 min) to -2.43 (60 min). These results indicate that the solutions still had the potential to further dissolve $CaSO_4$ and, since they were so undersaturated, it would be expected that any $CaSO_4$ phase present in the solutions will dissolve at a rapid rate. Given that the release of Ca (and SO_4) slowed down considerably after 10 min (as seen in *Fig. 4-7*), it is reasonable to conclude that most of the $CaSO_4$ surface coating on the ash particles has almost completely dissolved after 60 min.

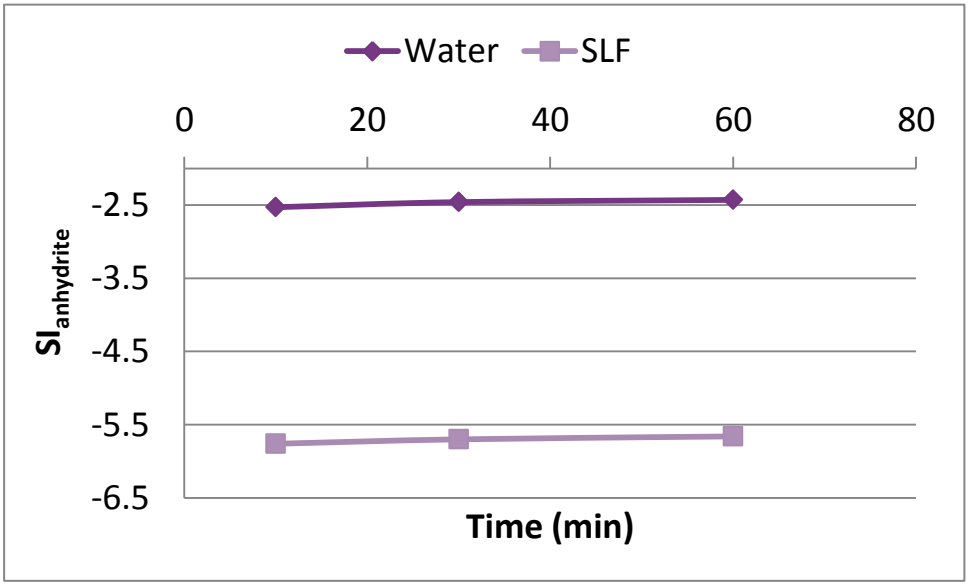

Figure 4-8: Evolution of $CaSO_4$ (anhydrite) saturation index (SI) with time in water and SLF as modelled by PHREEQC (Parkhurst and Appelo, 2013). The simulation was done using the water leach data for $CaSO_4$_G sample.

A similar result would be expected to occur if the same particles were dissolved in SLF, as can also be seen from the results of the modelling (*Fig. 4-8*). Since Ca and SO_4 are already present in SLF it could be expected that it would not dissolve $CaSO_4$ as efficiently as pure water, however the SLF solution itself is well undersaturated on $CaSO_4$, with a calculated saturation index of -6.97. Furthermore, even if the same amount of Ca, Na, Mg and SO_4 observed in the water leaching experiments (*Fig. 4-7*) are added to the SLF solution (*i.e.*, assuming that an equal amount of ash in contact with SLF will result in the complete dissolution of the

$CaSO_4$ surface coating), PHREEQC simulations indicate that the final solutions will still be highly undersaturated with respect to $CaSO_4$, with a final saturation index of -5.66.

4.3.3 Volcanic ash nebulisation

The average cell-deposited doses of nebulized particles range between 0.48 – 0.69 µg/cm^2, with an overall mean deposition dose of 0.58 µg/cm^2 (*Fig. 4-9A*).

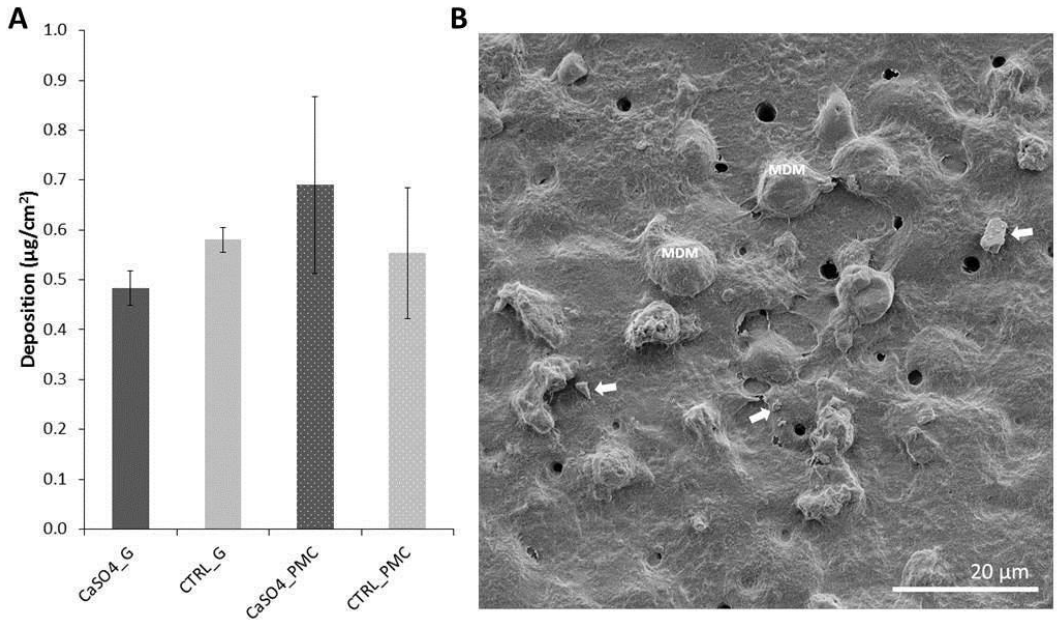

Figure 4-9: Deposition of nebulized respirable fraction of salt-laden and control glass and pumice particles. A) Average mass deposition (µg/cm^2) of salt-laden glass (CaSO4_G) and pumice (CaSO4_PMC) and their respective controls (CTRL_G and CTRL_PMC) quantified using a quartz crystal microbalance (QCM), following their dry nebulisation using a dry powder insufflator (DP-4, *Penn Century,* USA). Data are shown as the mean ± standard error of the mean, related to the following repetitions for each exposure: CaSO4_G and CTRL_G n=5, CaSO4_PMC and CTRL_PMC n=3. **B)** Representative scanning electron micrograph of the top side of the cell culture membrane, with monocyte-derived macrophages (MDM), showing deposited ash particles (indicated with white arrows). The image was collected at 2 kV and 16.13 mm working distance. Scale bar is 20 µm.

4.3.4 Particle toxicity

For the assessed biological endpoints (cytotoxicity, oxidative stress and (pro-)inflammatory mediators, including measurements for both protein production and gene expression), no significant (p > 0.05) changes were observed following cell culture exposures to any of the particle type used.

Images acquired by LSM showed no changes in cell morphology when compared to the untreated cells, *i.e.*, negative control (*Fig. 4-10*). The release of LDH by the cells did not differ significantly ($p > 0.05$) from the negative control for different particle type treatments, while it was significantly increased for the positive assay control Triton-X ($p < 0.05$), confirming the responsiveness of the biological model used for the measured endpoint (*Fig. 4-11A*). Regarding the expression of pro-apoptotic genes *FAS* and *CASP7*, none of the exposures showed a statistically significant ($p > 0.05$) outcome (*Fig. 4-11B*). In addition, there was no apparent difference in gene expression between different particle types.

The GSH assay was conducted, as has been done in ***Chapter 5***, but the positive assay control *tert*-Butyl Hydrogen Peroxide (*t*BHP; 0.1-10 mM) either did not induce oxidative stress or was cytotoxic, in this particular batch of experiments, so the data could not be considered reliable (***Appendix 1***). In addition, due to the limited number of samples, only one time-point has been chosen in the study, although 1 h or 4 h time-points could be more optimal for measurements, before the potentially reduced GSH levels are recovered. However, the data on exact recovery times for the triple cell co-culture system are not available at the present. Yet, the measurement of expression of *HMOX1* and *NQO1*, genes related to oxidative stress, allowed for a reliable assessment of particle-induced oxidative stress. The analysis showed no significant ($p > 0.05$) increase of gene levels after particle exposures when compared to the negative control (*Fig. 4-12*).

Similarly, no significant (pro-)inflammatory response was observed for any test particle ($p < 0.05$), as measured for the chosen markers. Production of IL-8 was seen for all particles, however, the levels did not differ significantly from the negative control (*Fig. 4-13A*), whereas the levels of TNF-α and IL-1β were below the method detection limits (***Appendix 1***). The positive assay control, LPS, significantly increased the release of IL-8. In agreement with the protein measurements, none of the cell treatments induced a change in the mRNA levels of (pro-)inflammatory markers measured relative to negative control (*Fig. 4-13B*).

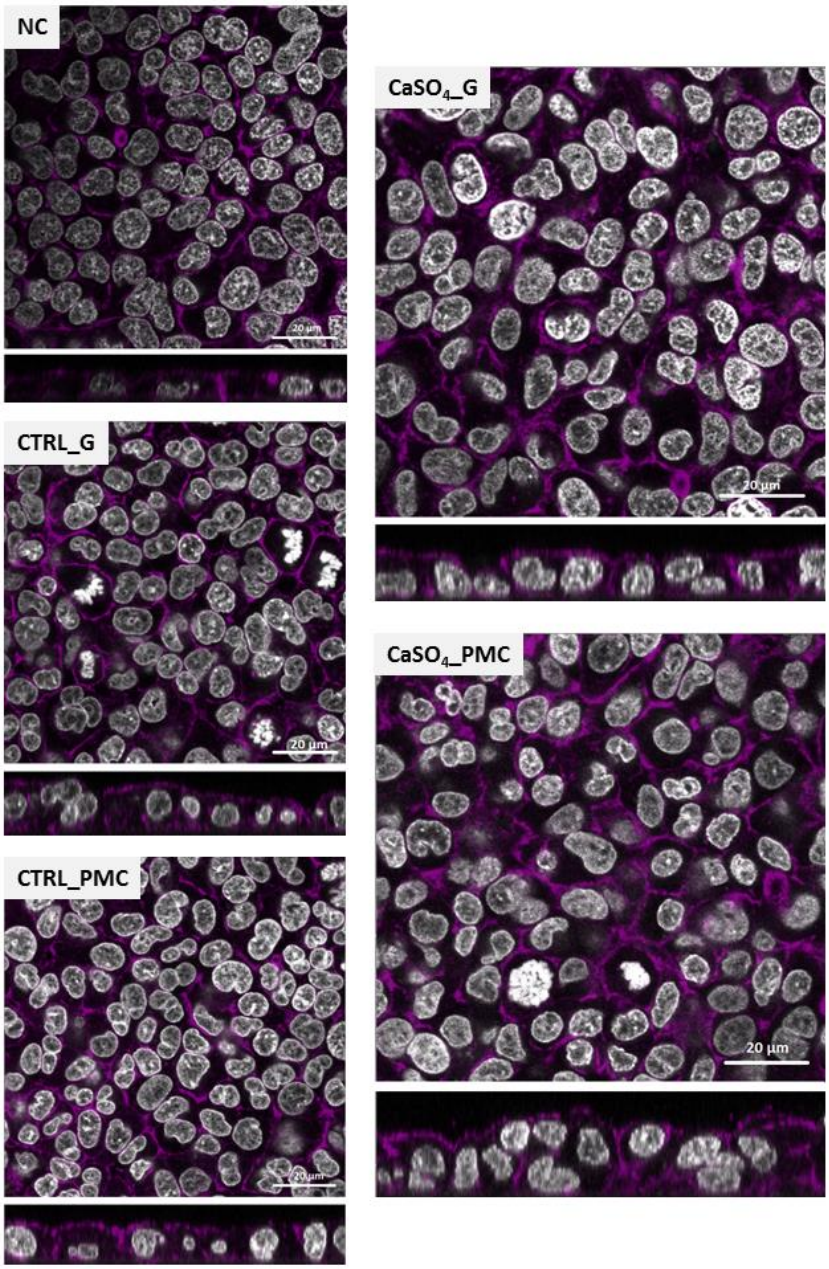

Figure 4-10: Cell morphology of the multicellular lung model following exposures to salt-laden glass and pumice particles. Representative confocal LSM images from XY and XZ projections for cultures exposed to salt-laden glass (CaSO4_G) and pumice (CaSO4_PMC) and their respective controls (CTRL_G and CTRL_PmmmMC). Cells were stained with Phalloidin-Rhodamine (F-actin cytoskeleton, magenta) and DAPI (cell nuclei, grey). Scale bars are 20 µm.

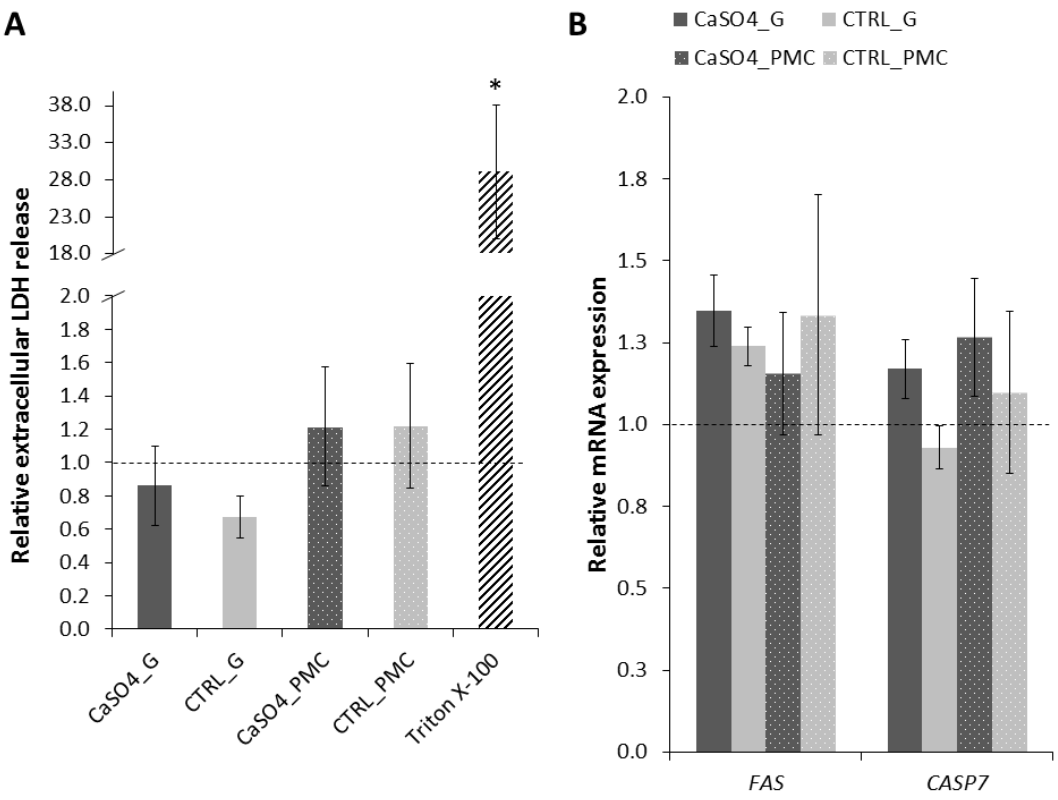

Figure 4-11: Cell viability of the multicellular lung model following exposures to salt-laden glass and pumice particles. **A)** Extracellular LDH levels in the culture medium normalized to negative control (cRPMI only). The positive assay control was 0.2% Triton X-100 in PBS. Data are presented as the mean ± standard error of the mean. The data shown are related to the following repetitions for each exposure: CaSO4_G and CTRL_G n= 5, CaSO4_PMC and CTRL_PMC n=3. * denotes significant difference ($p < 0.05$) between positive control and the other samples tested. **B)** Amounts of mRNA of pro-apoptotic genes FAS receptor (*FAS*) and caspase 7 (*CASP7*). Data are presented as the mean ± standard error of the mean, related to the following repetitions for each exposure: CaSO4_G n= 5, CTRL_G n=4, CaSO4_PMC and CTRL_PMC n=3.

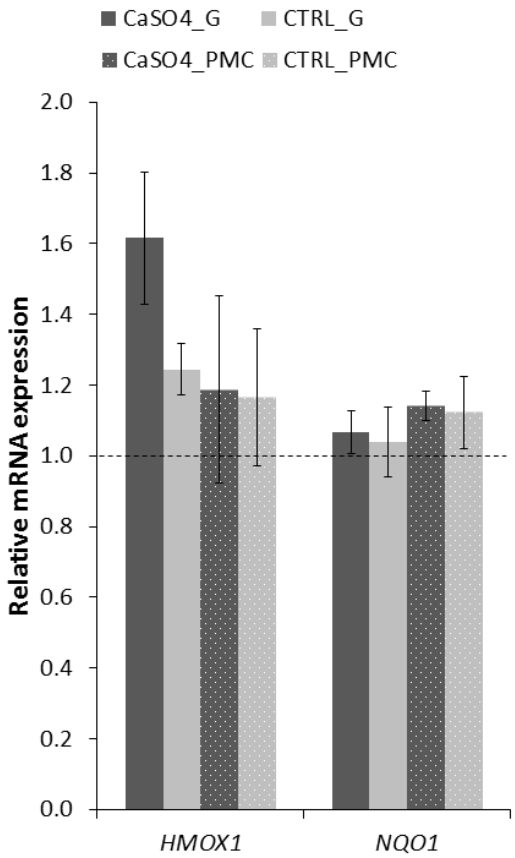

Figure 4-12: Oxidative stress response in the multicellular lung model following exposure to salt-laden glass and pumice particles. Amounts of mRNA of oxidative stress responsive genes, heme oxygenase 1 (HMOX1) and NAD(P)H dehydrogenase [quinone] 1 (NQO1). Data are presented as the mean ± standard error of the mean. The data shown are related to the following repetitions for each exposure: CaSO4_G n= 5, CTRL_G n=4, CaSO4_PMC and CTRL_PMC n=3.

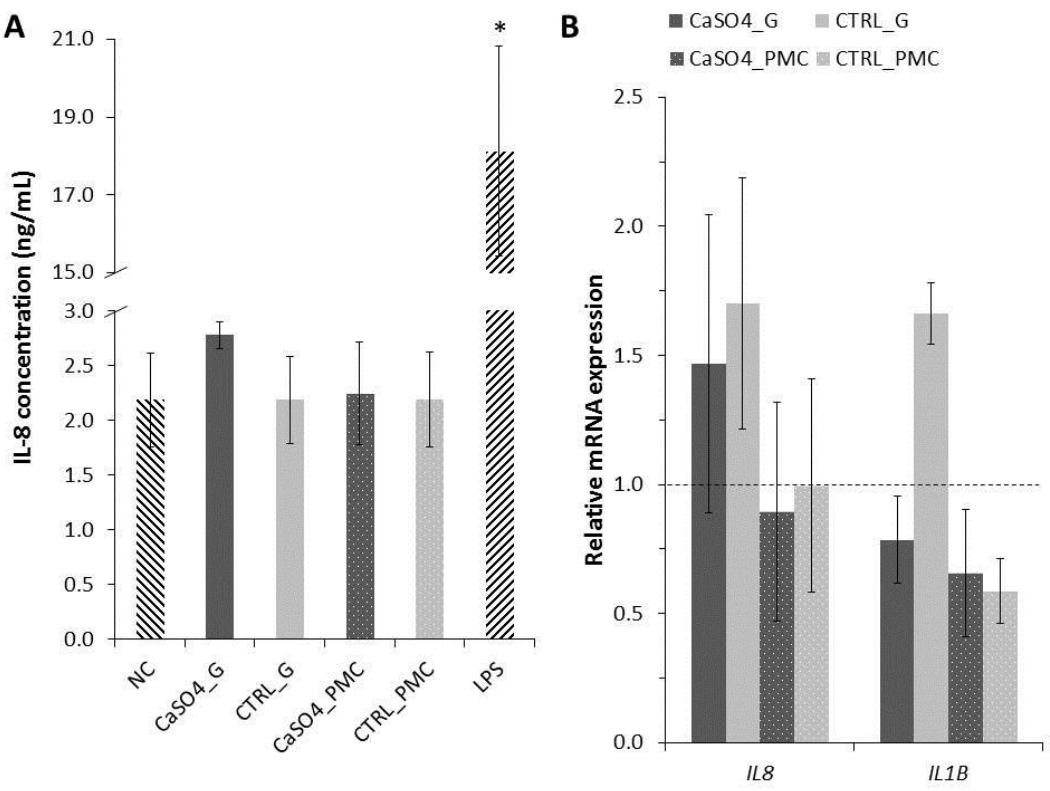

Figure 4-13: Release of (pro-)inflammatory mediators in the multicellular lung model following exposure to salt-laden glass and pumice particles. A) Interleukin-8 (IL-8) release in the culture medium following exposures to salt-laden glass (CaSO4_G) and pumice (CaSO4_PMC) and their respective controls (CTRL_G and CTRL_PMC). The positive assay control was lipopolysaccharide (LPS; 1 µg/mL). The negative control (NC) was untreated cells (cRPMI only). Data are presented as the mean ± standard error of the mean (n=3). * denotes significant difference ($p < 0.05$) between the positive control and the other samples tested. **B)** Amounts of mRNA of (pro-)inflammation-related genes encoding IL-8 (*IL8*) and interleukin-1 beta (*IL1B*). All data are presented as the mean ± standard error of the mean. The data shown are related to the following repetitions for each exposure: CaSO4_G n= 5; CTRL_G, CaSO4_PMC and CTRL_PMC n=3.

4.4 Discussion

This study is the first to investigate the impact of volcanic ash-plume interactions on the respiratory toxicity of volcanic ash. The results show that exposure of an *in vitro* multicellular model of the human lung to artificially generated pristine and salt-laden volcanic particulate did not induce any significant effects on any of the biological endpoints measured (cytotoxicity, oxidative stress and (pro-)inflammatory response).

4.4.1 In-plume processing of volcanic ash

Freshly erupted volcanic ash surfaces are composed of heterogeneously distributed chemically reactive surface sites, including acidic, basic, reduced and oxidised sites in various proportions (Maters et al., 2016). These sites readily react with gases and aerosols in the volcanic plume (Ayris et al., 2014, 2013), thus resulting in potential changes to surface chemistry and the formation of surface deposits (salts). Maters et al. (2016) found that in-plume processing likely modulates the nature and abundance of such reactive sites on particle surfaces, with volcanic ash generally comprising a lower fraction of basic sites and a higher fraction of oxidized sites (relative to acidic and reduced sites) when compared to volcanic glass. Consequently, such processing leads to partial removal/neutralization of sites available for further reactions, evident as well from findings of another study by Maters et al. (2017), which investigated the uptake of atmospheric trace gases on ash surfaces.

In the present study, a model system comprised of volcanic glass and pumice was used to understand whether gas-ash interactions affect particle reactivity *in vitro*. Following experiments in the AGAR, and in agreement with previous studies (Maters et al., 2017, 2016), volcanic glass was shown to be more reactive towards SO_2 than pumice, as evident from the abundance of SO_4 in the water leachate data (*Fig. 4-6*), which suggests a higher abundance of soluble S-bearing products on the glass surface resulting from the gas uptake. This can be attributed to the differences between the model particles, where pumice constitutes not only amorphous glass

but, also, mineral phases in various proportions, thus offering a different array of reactive sites for gas uptake (Maters et al., 2016).

In leachate solutions of both salt-laden samples, the concentrations of Ca^{2+} and SO_4^{2-} were dominant, with volcanically relevant quantities, with $CaSO_4$_G leachable concentrations (mg/kg) comparable to mean concentrations from a global dataset (Ayris and Delmelle, 2012) (see *Table 4-2*). The results suggest that the dominant reaction product following exposures in the AGAR was $CaSO_4$, which is in congruence with earlier experiments investigating scavenging of SO_2 by glass surfaces of varying bulk compositions (Ayris et al., 2013). The presence of these elements on the ash surface, as $CaSO_4$ salt, is usually confirmed from stoichiometric calculations of Ca^{2+} to SO_4^{2-} ratios in water leachate, which should be approximately 1:1 (Rose et al., 1973, Rose, 1977, De Hoog et al., 2001). Here, the Ca^{2+}/SO_4^{2-} ratios were 0.7 and 0.8 for salt-laden glass and pumice, respectively, indicating that, in addition to $CaSO_4$, different sulphate salts must also have been formed. The likely candidate is a Na_2SO_4, which has been shown to co-exist with $CaSO_4$ at 300-600 °C, as identified from leaching temperature-series experimental glass powders (Ayris et al., 2013). Since some Mg^{2+} was also measured in the leachate, another potential compound could be $MgSO_4$, a typical surface deposit found on pristine volcanic ash as well (Olsson et al., 2013, Bagnato et al., 2013, De Hoog et al., 2001, Rose, 1977).

Whilst in the present experiments Ca^{2+} and SO_4^{2-} from the surface coatings are the most abundant species available to interact with the *in vitro* lung model, it has to be taken into account that natural volcanic ash may exhibit variabilities in the nature and abundance of salt content (Olsson et al., 2013, Ayris and Delmelle, 2012, Witham et al., 2005). For example, ash generated through disruption of a hydrothermal system is likely to be much higher in salt precipitates than magmatic-generated samples (Witham et al., 2005). The post-AGAR samples lacked typical Cl-bearing salts, such as NaCl, $CaCl_2$ and $MgCl_2$, as HCl was not used during the SO_2 experiments, and confirmed by the low release of soluble chloride in the water leach (which was found to be below the detection limit of the method, with the exception of the control pumice sample: 53.7 ± 2.7 mg/kg of Cl^-). This was also confirmed with SEM imaging, since there was no evidence of NaCl presence on the

particle surface (*Fig. 4-5*). Thus, any biological responses elicited in the *in vitro* model exposed to glass and pumice in the current study are discussed with regards to the presence of sulphate salts.

4.4.2 The effects of in-plume processing on ash toxicity *in vitro*

The role of particle surface chemistry in the pathogenesis of disease has been extensively studied, considering that the particle surface is the first to be in contact with the lung structures at the site of deposition, including cells and the lung lining fluid.

The lack of significant impact on cell viability, as measured by the release of LDH and the expression of pro-apoptotic genes *FAS* and *CASP7*, and low oxidative stress potential of all particles, is in agreement with previous research investigating ash toxicity *in vitro* (Cullen and Searl, 1998, Wilson et al., 2000, Damby et al., 2016, Damby et al., 2013, Horwell et al., 2013), as well as with other studies in this thesis (***Chapter 5*** and ***6***), despite the different substrates and application of coatings. The minimal (pro-)inflammatory response observed for all particle treatments is also largely in congruence with the aforementioned *in vitro* studies. In the following paragraphs, certain effects of the salt-laden samples are 'hypothesised' (since the observed changes are not statistically significant) and discussed to try to explain why the results did not support the *Hypothesis 1* (***Chapter 1***).

It was hypothesised that the salt-laden glass treatment ($CaSO_4$_G) might have impacted oxidative stress state. This treatment induced a non-significant ($p > 0.05$) response of *HMOX1*, an oxidative stress-related gene (*Fig. 4-12*). These findings would suggest that the antioxidant protective mechanisms have been switched on in response to the insult posed by particles, as was observed previously for macrophages coping with volcanic ash (Damby et al., 2016), despite minimal observed cytotoxicity. In addition, while non-significant ($p > 0.05$) and thus, not biologically relevant, exposure to this sample resulted in an increase in IL-8 production by the cells compared to the negative control, at both protein and gene levels (*Fig. 4-13*). Such response could be a consequence of the observed changes in oxidative stress status, as inflammation may be a result of increased production

of oxidants by macrophages upon ingestion of particles. To elucidate whether there is an association between the 'observed' responses, *e.g.*, the activation of oxidative stress-responsive transcription factor NF-κB, a protein complex responsible for IL-8 transcription and involved in regulation of the immune response to particles (Rahman and MacNee, 1998, Schins and Donaldson, 2000), could be measured.

To get an insight into the likely mechanism underlying such potential response, it was necessary to understand whether the salts would be taken into cells while still on the particle surface, or whether they would dissolve upon contact with cell surfactant prior to particle uptake. Phagocytosis, *i.e.*, the uptake of particles by phagocytic cells such as macrophages, is a relatively rapid process where particles may be engulfed within 10 min of making contact with the cell (*e.g.*, Paul et al., 2013). This means that particles deposited in the vicinity of a macrophage could have been taken up within the first 10 min following exposure, but it is likely that others were phagocytosed at a later point during the experiment. Therefore, 10 minutes was considered the minimum duration for particles to interact with lung fluid. The dissolution rate experiments showed that the majority of soluble Ca^{2+} and SO_4^{2-} are leached from the particle surface after only 10 min contact time with deionized water (*Fig. 4-7*). Dissolution modelling with PHREEQC (*Fig. 4-8*) showed that the SLF solution is undersaturated with respect to sulphates, indicating that the same amount of salt coatings would dissolve in SLF. Thus, it is likely that the dissolution of salt coatings occurred prior to particle uptake. This implies that the particles were ingested without the presence of salt coatings, with soluble compounds being dispersed and diluted in the surfactant.

Therefore, it is necessary to discern whether the dissolution of salt coatings might have had a role in the assumed effects. While exposure to salt-laden glass non-statistically affected the normal redox state of the cell culture and IL-8 production, salt-laden pumice did not incite such a response. One reason for this could be that the lower soluble salt burden of pumice sample ($CaSO_4$_PMC), compared to $CaSO_4$_G, was not sufficient to elicit a response. The amount of $CaSO_4$ on glass was 3.95 x 10^{-3} µg/cm^2, whereas the amount of $CaSO_4$ on pumice was 1.39 x 10^{-3} µg/cm^2. Although the amount of $CaSO_4$ on glass was 3x higher, it is reasoned

that a slight increase in Ca^{2+} and SO_4^{2-} dissolved in the already heavily buffered surfactant would have a negligible impact on the chemistry of the solution. Both Ca^{2+} and SO_4^{2-} are important molecules required for normal function of the cells and are able to diffuse across the cell membranes if required by the cells (Yang and Hinner, 2015). In turn, the excess of such ions may alter cell homeostasis and activate various complex signalling pathways (*e.g.*, calcium signalling) and in this way, potentially induce toxicity (*e.g.*, Brown et al., 2004, Donaldson et al., 2003). For example, a study by Könczöl et al. (2012) found that $CaSO_4$ particles (applied as a suspension in cell medium and thus, dissolved to a considerable extent) were able to mediate ROS generation and induce DNA damage in A549 cells *in vitro*, but at doses as high as 100 µg/cm^2. In comparison, previous studies, on V79-cells (Chinese hamster lung cells) or IMR90-cells (human lung fibroblasts), have used $CaSO_4$ as a negative particle control and did not observe any toxic effects for doses < 20 µg/cm^2 (Dopp et al., 2005, Geh et al., 2006). When compared to these studies, the amount of Ca^{2+} and SO_4^{2-} dissolved from glass and pumice particles in the present study is negligible. Furthermore, the finding that the response to salt-laden and control pumice samples was comparable, across all measured biomarkers, further supports the notion that salts do not have a significant role in the observed effects. As such, it is difficult to attribute the observed response to $CaSO_4$_G to the presence of the salt coating.

Exposure to the glass control sample (CTRL_G) caused relatively high, but non-significant ($p > 0.05$) expression of both *IL8* and *IL1B* than the negative control and other particle treatments (*Fig. 4-13B*). The first reason for this could be the potential difference in surface reactivity of samples. It is possible that such surfaces may have bound proteins released by the cells more effectively and thus resulted in an underestimation of cytokine production following exposure to these particle types (Brown et al., 2010, Gasser et al., 2012), since no proteins were measured at a corresponding level in the supernatant. This could be tested using a bioreactivity assay to determine the strength of binding between particles and lung proteins, as previously done for volcanic ash by Jones and BéruBé (2011). The second possibility could be that the main driver of the response to both glass samples could be the

substrate itself, and that the presence of surface salts may have diminished the potential for a similar level of (pro-)inflammatory response (that is, *IL1B* upregulation) in $CaSO_4$_G sample, although this is difficult to state due to the lack of significant effect observed.

Regarding *Hypothesis 1* (***Chapter 1***), the toxicity of ash does not appear to be altered by the presence of a salt coating. Although, how salts are being processed by the surfactant, once removed from the particles surface, needs to be clarified. However, the observation that volcanic particle substrate (glass) has a potential to initiate an inflammatory response suggests that the alterations that resulted from particle processing in the AGAR may have affected its biological reactivity in the current experimental conditions. As discussed earlier in the chapter, it has been shown that in-plume processing affects the nature and abundance of available reactive sites on particle surfaces, and surface chemical properties have been previously linked with adverse biological effects of particle exposures (Fubini et al., 1995a, Setyan et al., 2010). The potential differences in surface properties originating from eruptive plume processing may thus represent the key for understanding the observed variability in volcanic ash toxicity (reviewed in Baxter et al., 2014, Horwell and Baxter, 2006) and deserve further attention.

4.5 Conclusion

This study provides the first insight into the potential biological impact of volcanic ash in the presence or absence of surface salt coatings, by constraining the reactions of volcanic ash in an eruptive plume in a laboratory setting and measuring the related changes that may affect its toxicity *in vitro*.

The findings show that the presence of surface soluble sulphate coatings has a limited adverse biological impact to cells in an acute scenario, considering the biological endpoints measured. Emplaced surface salts are likely to dissolve in the lungs prior to the uptake by phagocytic cells, and it is reasoned that a slight increase in Ca^{2+} and SO_4^{2-} dissolved in the already heavily buffered surfactant would

have an insignificant impact on the chemistry of the solution.

The results suggested that the studied in-plume processes likely do not affect the toxicity of volcanic ash, however, further research should investigate different types of gas-ash interactions, *e.g.*, different volcanic gases and at different magmatic temperatures, including associated changes to surface chemical functionalities and whether they can be correlated with biological responses *in vitro*.

The observations of this study advance the understanding of the hazard posed by volcanic ash and, specifically, the impact of in-plume processing on its toxicity, thus providing valuable information for health risk assessment in future volcanic eruption events.

4.6 References

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Part III

The urban environment and its impact on the respiratory toxicity of volcanic ash

Chapter 5

An *in vitro* study of the potential respiratory hazard of combined exposure to volcanic ash and diesel exhaust particles

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5.1 Introduction

Communities resident in urban areas located near active volcanoes can experience volcanic ash exposures during, and following an eruption, in addition to sustained exposures to high concentrations of anthropogenic air pollutants (*e.g.*, vehicle exhaust emissions, with diesel exhaust particles (DEP) being one of the main constituents (WHO, 2013)). Inhalation of anthropogenic pollution is known to cause the onset of, or exacerbate, respiratory and cardiovascular diseases (see ***Chapter 2***, *Section 2.8*). It is postulated that similar exposure to volcanic ash can also affect such disease states (Baxter et al., 1983, Baxter et al., 1981). Yet, currently, limited understanding surrounds the human health hazard associated with the combined (inhalation) exposures that result from the addition of volcanic particulate matter (PM) to the urban environment.

The aim of this study, therefore, was to investigate, for the first time, the biological impact of concomitant exposure to volcanic ash (from Soufrière Hills volcano, Montserrat) and a model urban pollutant – standardised DEP sample (*National Institute of Standards and Technology's Standard Reference Material* (NIST SRM 2975) using a sophisticated multicellular *in vitro* model mimicking the human epithelial tissue barrier (Rothen-Rutishauser et al., 2005). Thus, the current study provides a landmark first assessment of these issues, the findings of which are highly relevant for volcanic health hazard management on a global scale.

Being the first study of its kind, a simple, standardised analogue particle was employed (DEP) to gain a first understanding of the potential for the combined reactivity of ash and urban pollution in the lung, without the complexity of heterogeneous particles or gas-particle mixtures. DEP toxicity has been previously well-investigated and established (Clift et al., 2014a, Steiner et al., 2016).

5.2 Methods

In vitro experiments were conducted using a sophisticated 3D triple cell co-culture model of the human alveolar epithelial tissue barrier (***Chapter 3***, *Section 3.5.1*). A dry powder insufflator (***Chapter 3***, *Section 3.5.2.1*) was used to nebulise the dry, respirable fraction (isolated as described in ***Chapter 3***, *Section 3.3.1*) of the volcanic ash from Soufrière Hills volcano, Montserrat (see ***Chapter 3***, *Section 3.2.1*) for direct deposition onto the cell cultures at the air-liquid interface (ALI). Initial dose-dependent and dose-response analysis were performed to determine the optimal dose to use for volcanic ash in the cell exposure experiments (***Chapter 3***, *Section 3.5.2*).

Key health-pertinent characteristics of volcanic ash, namely particle size distribution and specific surface area, were characterised using laser diffraction and the BET method, respectively (as described in ***Chapter 3***, *Section 3.4*).

The cell cultures were individually exposed to respirable volcanic ash or DEP (see *Section 5.2.1*) for a period of 24 h at the ALI and at the pseudo-ALI, respectively (see *Section 5.2.2*). Subsequently, co-cultures were co-exposed to both particle types (*Fig. 5-1*). The biological impact of each individual particle type and co-exposures was analysed to investigate common particle-induced toxicity mechanisms (described in ***Chapter 2,*** *Section 2.3.4*). The cytotoxicity (LDH release), oxidative stress (depletion of intracellular GSH) and production of (pro-)inflammatory markers (TNF-α, IL-8 and IL-1β) were assessed after the exposures. The impact of volcanic ash exposure upon cell morphology, as well as its interaction with the multicellular model, was visualised via confocal laser scanning microscopy (LSM) and scanning electron microscopy (SEM), respectively. Additional information about the bioanalysis assays performed is outlined in ***Chapter 3***, *Section 3.5*.

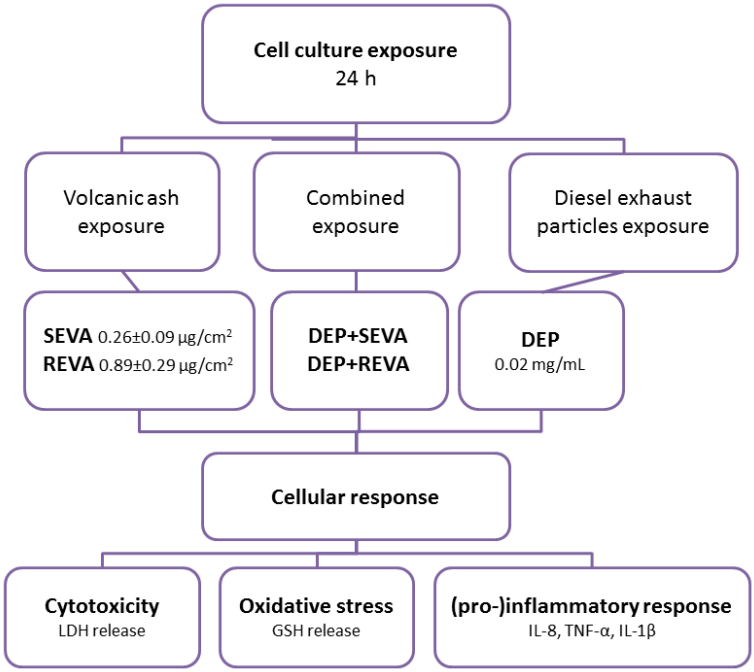

Figure 5-1: Scheme of the experimental design of this study, where the triple cell co-culture model and the dry powder insufflator were used for the first time in a volcanic ash toxicity *in vitro* study. SEVA = single exposure to volcanic ash, REVA = repeated exposure to volcanic ash, DEP = diesel exhaust particles, DEP+SEVA = co-exposure to diesel exhaust particles and 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash, and DEP+REVA = co-exposure to diesel exhaust particles and 0.89 ± 0.29 µg/cm^2 of repeated exposure to volcanic ash.

5.2.1 Diesel Exhaust Particles

As a model urban pollutant, standard DEP (NIST SRM 2975), collected from an industrial diesel-powered forklift, was used (NIST, 2013). The key characteristics of this standard sample have been reported in the material's Certificate of Analysis (NIST, 2013) and are summarised in the Results section (*Section 5.3.1*).

For DEP, a quasi-ALI approach (see *Section 5.2.2.2*) was adopted as it was not possible to nebulise DEP with the dry powder insufflator device, due to the increased electrostatic nature of DEP in dry powder form. In the attempts to use the insufflator, it was found that DEP did not discharge evenly over the cell culture plate and their deposition (*i.e.*, mass) could not be detected by the quartz crystal microbalance (QCM). Further, when pre-mixed with volcanic ash sample, DEP were observed to electrostatically stick to, and cover, the ash surfaces (*Fig. 5-2*), thus creating clumps. It was assumed this would have an impact on the discharge

pattern of particles, and hence, ash and DEP could not be applied concomitantly to the cell cultures as a dry powder mixture.

Instead, a suspension of DEP was prepared as follows; 1 mg of dry DEP was suspended in 1 mL cell culture medium RPMI 1640 (supplemented with 1 % L-Glutamine, 1 % Penicillin/Streptomycin and 10 % fetal bovine serum). The pre-mixed solution was subsequently sonicated for 90 min at 37 kHz at 37 °C. This stock suspension of DEP was diluted with supplemented RPMI 1640 medium to a working concentration of 0.02 mg/mL. Using this dose of DEP was based upon the findings previously shown by Clift et al. (2014a), who undertook a dose-response analysis of the same DEP sample upon the same co-culture system.

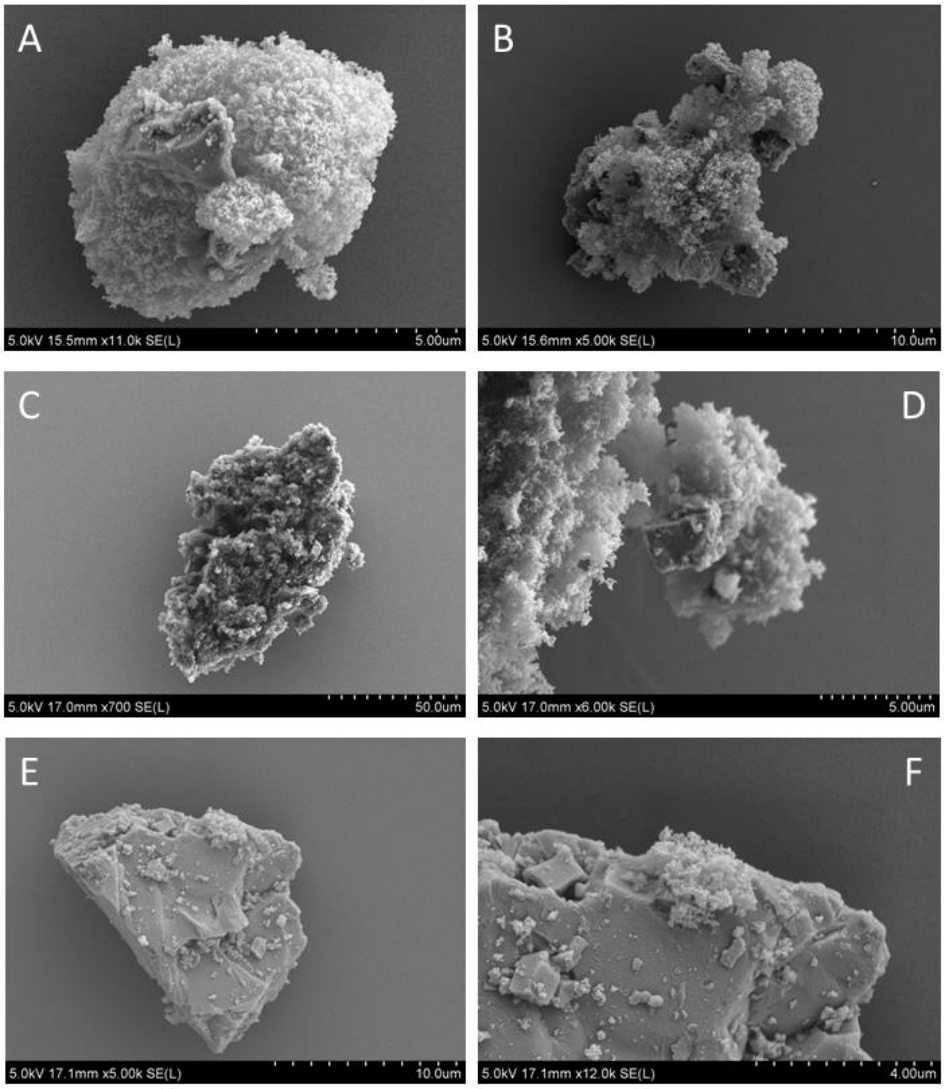

Figure 5-2: Representative SEM images of volcanic ash and diesel exhaust particles dry powder mixture. (D) is an inset of (C), and (F) is an inset of (E). Images were collected at 5.0 kV; WD (mm) and scale (μm) are noted on the images.

5.2.2 Cell culture exposures

5.2.2.1 Cell exposures to volcanic ash

Based upon the dose-response analysis (***Chapter 3***, *Section 3.5.2.3*), the highest mass (8 mg) was subsequently chosen to be used in this study to assess the biological impact towards the triple cell co-cultures as a single exposure (SEVA), representing a 'low dose' (0.26 ± 0.09 µg/cm^2) and 3x this highest mass - repeated exposure (REVA) - representing a 'high dose' (0.89 ± 0.29 µg/cm^2) of volcanic ash (*Fig. 5-1*, *Fig. 5-3*). Ash was nebulised directly over the cell cultures at the ALI using a dry powder insufflator (as described in ***Chapter 3***, *Section 3.5.2.1*).

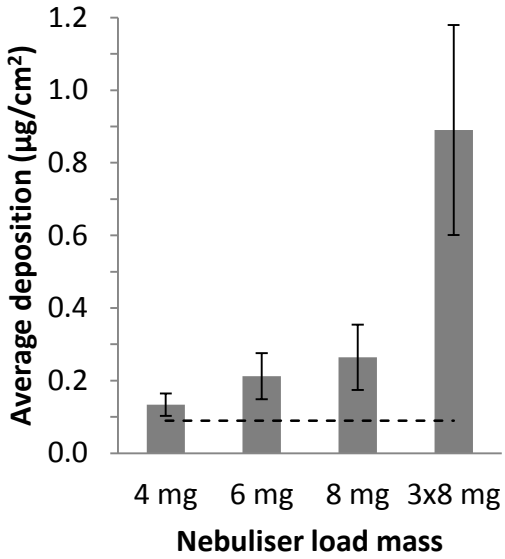

Figure 5-3: Deposition of the nebulized respirable fraction of volcanic ash as determined by the dose-dependent analysis (***Chapter 3***, *Section 3.5.2.2*). The corresponding average dose (± standard error of the mean) that was deposited onto the cells was 0.13 ± 0.03 (n = 14), 0.21 ± 0.06 (n = 14) and 0.26 ± 0.09 µg/cm^2 (SEVA; n = 17) for 4, 6, and 8 mg starting (feed) mass, respectively. The average mass deposited in the repeated exposure scenario (3x8 mg) was 0.89 ± 0.29 µg/cm^2 (REVA; n = 9). The dashed line is the threshold limit for the QCM measurement (0.09 µg/cm^2).

5.2.2.2 Cell exposures to DEP

As mentioned, DEP were used in a pseudo-ALI exposure experiment, where a total volume of 100 µl of DEP at 0.02 mg/mL suspended in the supplemented medium was added to the apical compartment of the triple cell co-culture model at the ALI. When deposited from the suspension, onto the cells grown on a 4.2 cm^2 surface

insert, applied DEP would equate to a deposited dose of 0.5 µg/cm^2. This dose falls in the range of ash deposited doses and is close to an average between the low and high ash dose.

5.2.2.3 Combined exposures to DEP and volcanic ash

In the scenario of combined exposures to DEP and ash, directly after the exposure to DEP (as in *Section 5.2.2.2*), the load mass of volcanic ash (8 mg) was nebulised over the cell cultures as described above in *Section 5.2.2.1*, either as a single exposure (SEVA) or repeated exposure (REVA) (*Fig. 5-1*).

5.3 Results

5.3.1 Particle characterisation

Particle size analysis of an isolated respirable fraction (***Chapter 3***, *Section 3.3.1*) from the Soufrière Hills ash sample showed that all particles were <10 µm (*Fig. 5-4*). The sample consisted of 12.2, 41.5 and 72.5 volume % of particles with sizes of <1, <2.5 and <4 µm, respectively. Specific surface area, determined by the Brunauer-Emmett-Teller (BET) (Brunauer et al., 1938) analysis with nitrogen adsorption (***Chapter 3***, *Section 3.4.4*), was 3.2 m^2/g.

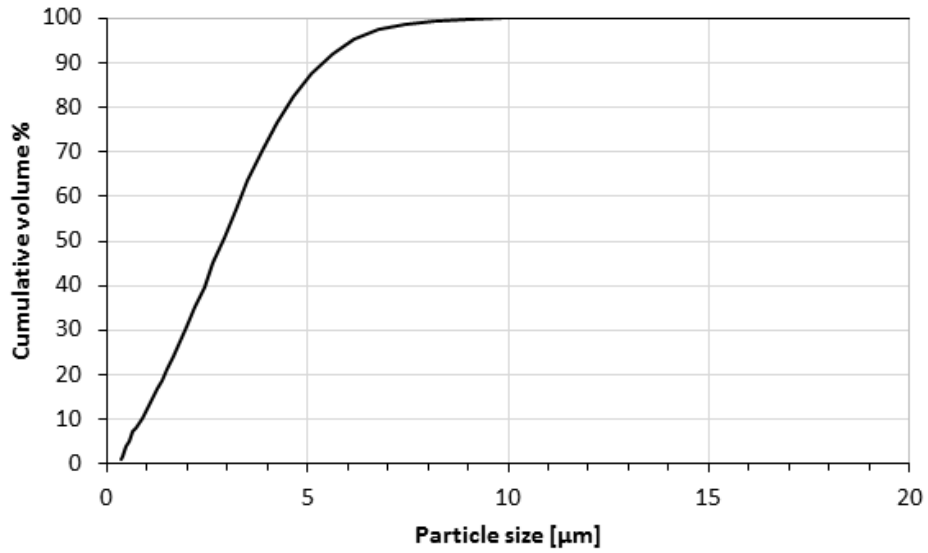

Figure 5-4: Particle size distribution of the isolated respirable fraction of Soufrière Hills volcanic ash.

Characteristics for the DEP (SRM 2975) used were reported in NIST (2013). Briefly, they had a mean diameter (by volume distribution) of 31.9 ± 0.6 µm, with a median diameter (*i.e.*, 50 % of the volume is less than) 19.4 ± 0.3 µm. The specific surface area of the DEP was 91 m^2/g determined by nitrogen adsorption measurements following the BET method.

5.3.2 Cellular response to particle exposures

5.3.2.1 Interaction of volcanic ash with triple cell co-cultures

SEM images of the upper surface of the triple cell co-culture exposed to dry ash at the ALI showed that ash was able to get in contact with and be engulfed by the macrophage cache of the co-culture system (*Fig. 5-5E and F*). Ash was as well observed on the lower surface of the triple cell co-culture membrane, in direct contact with the dendritic cells (*Fig. 5-5A, B, C and D*).

5.3.2.2 Exposure to volcanic ash

As determined *via* the release of the cytosolic enzyme lactate dehydrogenase (LDH), no significant cytotoxicity ($p > 0.05$) was observed after 24 h following exposure to either SEVA (0.26 ± 0.09 $\mu g/cm^2$; n=4) or REVA (0.89 ± 0.29 $\mu g/cm^2$; n=3) compared to the negative control (defined as supplemented cell culture medium from the untreated cells) (*Fig. 5-6A*). The lack of any cytotoxicity associated with the SEVA and REVA exposure upon the cell cultures was qualitatively supported by the observation that no alteration to cellular morphology occurred, as visualised by LSM (*Fig. 5-7*). It was further observed, by LSM, that the epithelial layer was tightly bound together, forming a monolayer, with cells undergoing mitosis, suggestive of normal homeostasis (indicated with white arrows in *Fig. 5-7*).

Further assessment of the biochemical impact of SEVA and REVA upon the triple cell co-culture showed no significant ($p > 0.05$) loss in total reduced glutathione (GSH), a key indicator of oxidative stress *in vitro* (Clift et al., 2010) (*Fig. 5-6B*). Similar, negative effects were also observed for the ability for either SEVA or REVA to elucidate a (pro-)inflammatory response, with no significant ($p > 0.05$)

production of tumour necrosis factor α (TNF-α) or interleukin 8 (IL-8) after 24 h exposure (*Fig. 5-6C and D*). Unfortunately, for SEVA and REVA (alone, without DEP), there was insufficient volume of supernatant to analyse release of an additional, relevant (pro-)inflammatory marker, interleukin 1β (IL-1β) (*Fig. 5-8*).

It is important to note that, alongside these negative datasets, all positive controls used (*i.e.*, Triton X-100 (Triton; LDH assay), *tert*-Butyl Hydrogen Peroxide (*t*BHP; GSH assay) and lipopolysaccharide (LPS; TNF-α and IL-8)) incited significant effects within the assays, indicating that the biological model used was responsive for all endpoints (*Fig. 5-6*).

5.3.2.3 Exposure to DEP

Similar findings were observed following exposure of the co-culture to DEP alone, with no significant cytotoxicity (*Fig. 5-6A and 5-7*) or changes ($p > 0.05$) to the oxidative stress status of cells observed (*Fig. 5-6B*). Again, the positive assay control, *t*BHP, showed a significant depletion of GSH in the co-culture system, confirming the observation that the array of sample exposures incited no oxidative stress. Despite these findings, the DEP-only exposure to the *in vitro* multicellular system elicited a significant ($p < 0.05$) IL-1β response compared to the negative control (*Fig. 5-8*), while TNF-α and IL-8 responses were non-significant ($p > 0.05$) (*Fig. 5-6C and D*).

5.3.2.4 Combined exposure to DEP and volcanic ash

Combined exposures to DEP and volcanic ash (DEP+SEVA, DEP+REVA) also resulted in no significant cytotoxicity (*Fig. 5-6A and 5-7*) or changes ($p > 0.05$) to the oxidative stress status of cells (*Fig. 5-6B*). It was observed, however, that although the combined exposures did induce a heightened (pro-)inflammatory response in the co-cultures for TNF-α and IL-8 ($p > 0.05$) (*Fig. 5-6C and D*), only a significant ($p < 0.05$) release of IL-1β, compared to the negative control, was found (*Fig. 5-8*).

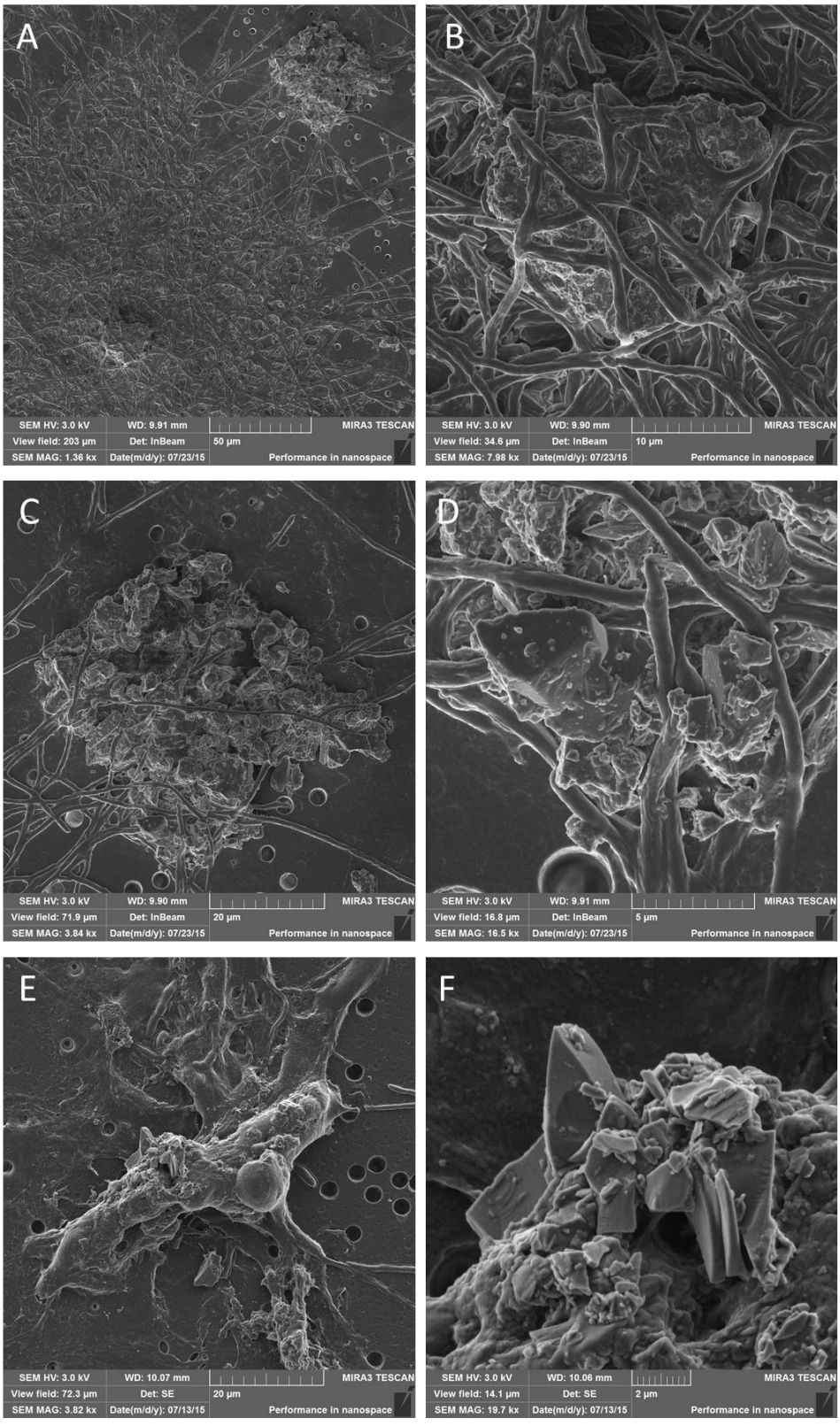

Figure 5-5: Interaction of volcanic ash with the triple cell co-culture. Scanning electron micrographs of the triple cell co-culture membrane exposed to 0.26 ± 0.09 µg/cm^2 of a single exposure to volcanic ash (SEVA), showing a direct interaction of ash particles with the different cell types. **A)** is showing the basal side of the triple cell co-culture with dendritic cells. **B)** and **C)** are the insets of the image **A)**, while **D)** is an inset of **C)**. Image **E)** is showing apical side of the cultures, with macrophage interacting with volcanic ash. **F)** is an inset of image **E)**.

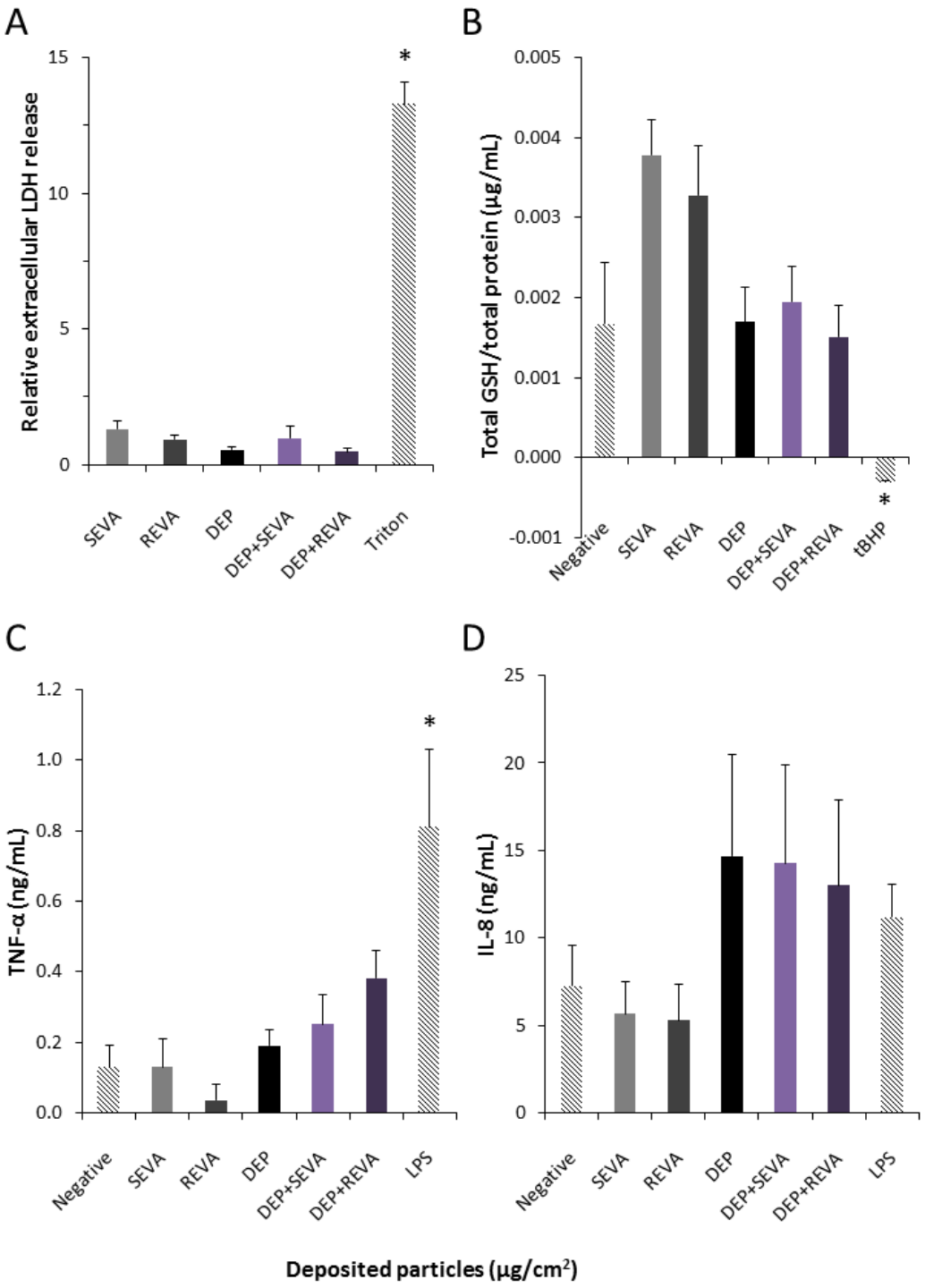

Figure 5-6: Biochemical response of the triple cell co-culture system following exposures to volcanic ash and diesel exhaust particles. A) Cytotoxicity presented relative to the negative control (cell culture medium only), **B)** total reduced glutathione (GSH), **C)** tumour necrosis factor-α (TNF-α), and **D)** interleukin-8 (IL-8) release of the triple cell co-culture model after exposure to 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash (SEVA), 0.89 ± 0.29 µg/cm^2 of repeated exposure to volcanic ash (REVA), diesel exhaust particles (DEP; 0.02 mg/mL), co-exposure to diesel exhaust particles and 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash (DEP + SEVA), and co-exposure to diesel exhaust particles and 0.89 ± 0.29 µg/cm^2 of repeated exposure to volcanic ash (DEP + REVA). Data are presented as the mean ± standard error of the mean (n=8 for positive and negative controls; n=4 for SEVA, n=3 the rest of the treatments). * denotes significant difference ($p < 0.05$) between negative control and the other samples tested.

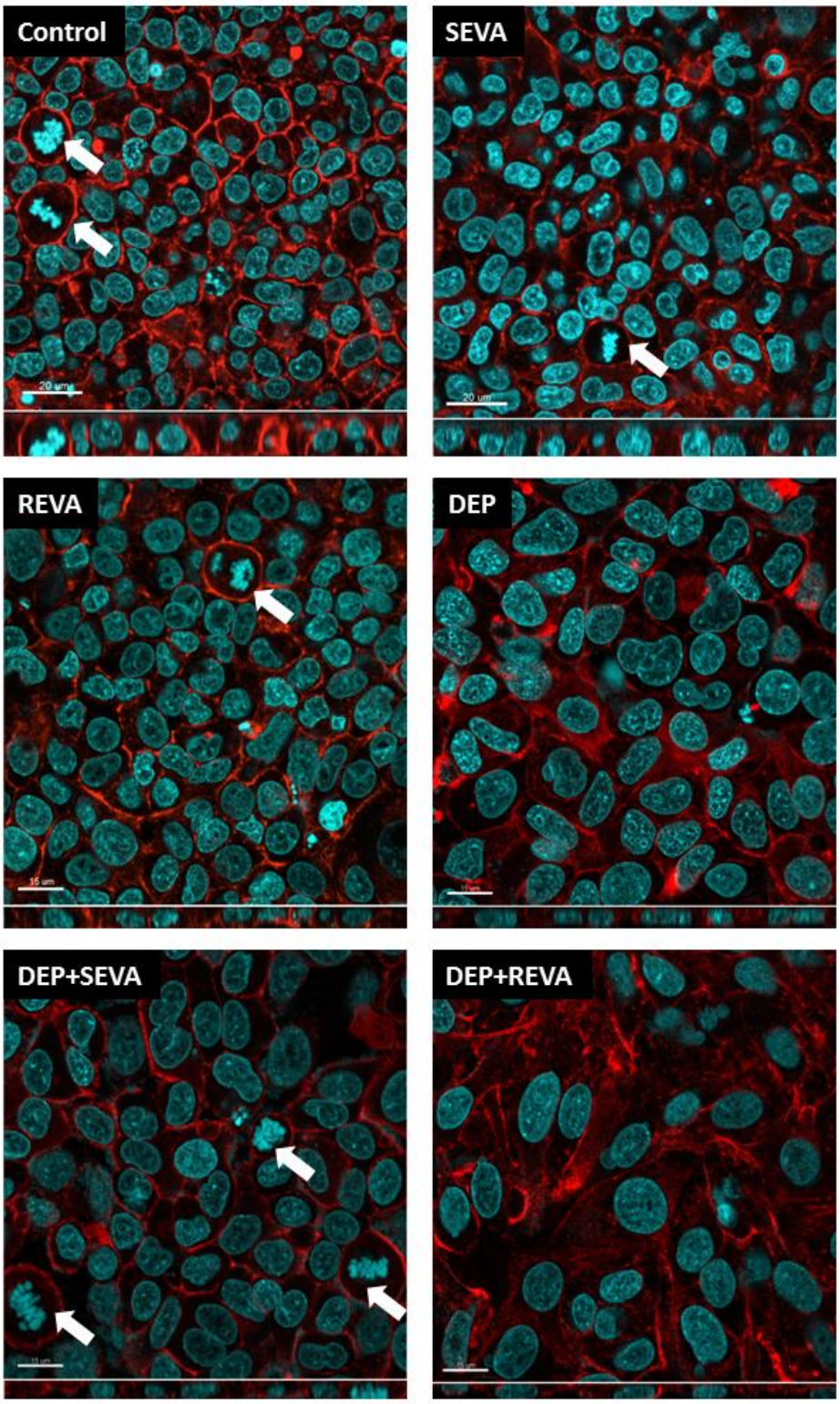

Figure 5-7: Cell morphology of the triple cell co-cultures exposed to volcanic ash and diesel exhaust particles. LSM images show the triple cell co-culture stained for F-actin cytoskeleton (red) and the nuclei (blue) for the control (unexposed) sample and following exposures to 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash (SEVA), 0.89 ± 0.29 µg/cm^2, repeated exposure to volcanic ash (REVA), diesel exhaust particles (DEP; 0.02 mg/mL), DEP and 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash (DEP + SEVA), and DEP and 0.89 ± 0.29 µg/cm^2 of repeated exposure to volcanic ash (DEP + REVA). White arrows indicate cells undergoing cell division. Scale bars are 20 µm (control and SEVA) and 15 µm (the rest of the images). Images were collected at x63 magnification.

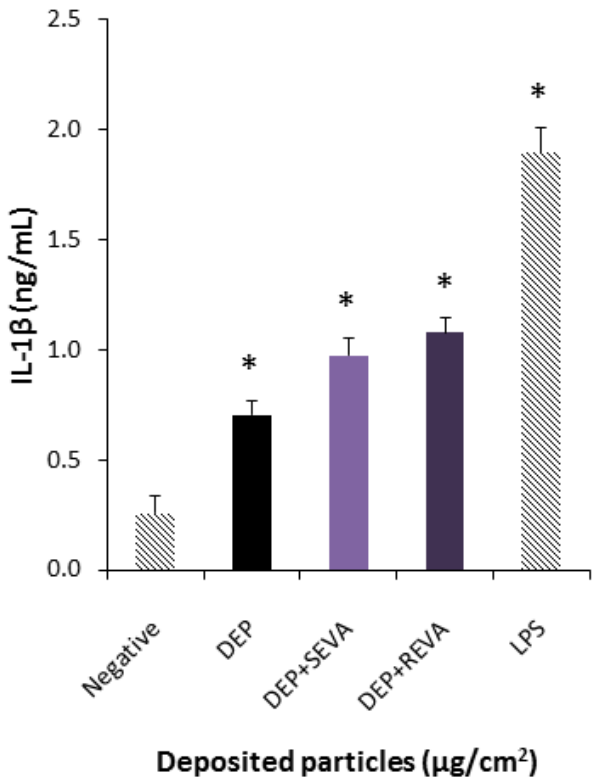

Figure 5-8: Interleukin 1β (IL-1β) release of the triple cell co-culture model after exposure diesel exhaust particles (DEP; 0.02 mg/mL), co-exposure to diesel exhaust particles and 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash (DEP + SEVA), and co-exposure to diesel exhaust particles and 0.89 ± 0.29 µg/cm^2 of repeated exposure to volcanic ash (DEP + REVA). Data are presented as the mean ± standard error of the mean (n=3). * denotes significant difference ($p < 0.05$) between negative control and the other samples tested.

5.4 Discussion

The purpose of this study was to gain a first understanding of the potential hazard of a combined volcanic ash and DEP exposure to the respiratory system by using a state-of-the-art *in vitro* approach.

To date, all previous *in vitro* studies performed on volcanic ash have used monoculture cell models, where the cell cultures have been immersed in cell medium and ash particles added, already suspended (pre-mixed sample), in a liquid medium. Whilst this approach is commonplace, it does not adequately reflect the physiological condition of a respiratory exposure to particles. In this thesis, the nebulisation method of ash using the dry powder insufflator (***Chapter 3***, *Section 3.5.2.1*) has enabled, for the first time in *in vitro* studies, application of volcanic ash

to cells at the ALI in its pristine, dry state. This method represents a more realistic scenario in comparison to all previous studies which suspended ash in cell medium. Furthermore, it has recently been shown that multicellular models can be useful tools in determining the specific (pro-)inflammatory and oxidative stress effects of particles compared to monocultures (Clift et al., 2014a).

Notably, the present study is a first screening of potential adverse effects of combined exposure to volcanic ash and DEP, therefore a simple approach of comparing the effect of a combined exposure with the effects of individual particles at comparable concentrations and exposure duration was used (Cassee et al., 1998). The effects following exposures to individual particle types were considered, and single exposure data for volcanic ash and DEP are compared to previous investigations using both *in vitro* and *in vivo* experiments (see *Sections 5.4.3* and *5.4.4*).

5.4.1 Interaction of volcanic ash with triple cell co-cultures

As this was the first study to utilise a multicellular model in terms of the biological impact of volcanic ash *in vitro*, its interaction with the model has been visualised and confirmed *via* SEM (*Fig. 5-5*). The observation that the macrophages were in direct physical contact with ash was expected, due to their role in the clearance of foreign material *via* the process of phagocytosis. Ash particles, as seen on *Fig. 5-5E* and *F*, appear to be partially engulfed by a macrophage, clearly indicating that the cells were responsive to the presence of ash. Previous studies have also shown the capacity of macrophages to internalise volcanic ash (Damby et al., 2016, Monick et al., 2013), however, this could not be directly observed using this technique.

The contact of ash with epithelial cells was not completely unexpected either, due to the surface area that they cover in the alveolar epithelial airway barrier *in vitro* system (insert membrane is 4.2 cm^2 with $0.5x10^5$ epithelial cells seeded compared to $5x10^4$ macrophages seeded in the co-culture) (Blank et al., 2007, Rothen-Rutishauser et al., 2005). As such, epithelial cells must have contributed to the overall biochemical response of the model following reactions with ash.

It is worth noting that, to the best of the author's knowledge, this was the first time that dendritic cells have been considered in *in vitro* investigation of the biological impact of volcanic ash. The observed direct contact of the ash with the monocyte-derived dendritic cells can be hypothesized to occur either through (I) translocation of the ash particles *via* cell-cell interactions, as previously described for other particle types (Blank et al., 2007), (II) direct translocation through the pores of the micro-porous membrane insert (3 µm), or (III) deposition between the micro-porous membrane outer ridge and the side of the well of the six-well plate during the nebulisation process. Further research would be necessary to determine how volcanic ash becomes potentially available to interact, or not, with the dendritic cells of the co-culture system, such as through translocation studies previously performed with this 3D *in vitro* lung model (Rothen-Rutishauser et al., 2007, Lehmann et al., 2009, Endes et al., 2015), as well as to deduce what biological impact this interaction may potentially elucidate and whether it would differ to the outcome of the present study (*i.e.*, lack of the effects).

5.4.2 Volcanic ash *in vitro* doses and airborne concentrations

For volcanic ash, doses of 0.26 ± 0.09 µg/cm^2 (SEVA) and 0.89 ± 0.29 µg/cm^2 (REVA) were chosen for the experiments (with rationale discussed in *Section 5.2.2.1*). It is difficult to conclude how representative these doses are in relation to actual deposited doses received after inhaling volcanic ash, primarily due to the lack of reliable *in vivo* dosimetry data available (see ***Chapter 2***, *Section 2.5.1*). Furthermore, exposure does not correspond directly to deposited dose as the respiratory system will remove some of the inhaled material *via* the mucociliary escalator and expectoration (see ***Chapter 2***, *Section 2.3.2*).

However, some assumptions can be made to allow an estimate of airborne particulate concentrations, equivalent to the doses used in this study. Assuming a daily inhaled air volume of 25 m^3 and an alveolar lung surface area of 100 m^2, which correspond to a healthy, moderately-active adult (ICRP, 1994), and an alveolar deposition efficiency of about 10 % (Lahde et al., 2013), it can be estimated (Paur et al., 2011) that the doses used in this study correspond to average airborne

concentrations of 104 and 356 mg/m^3 for SEVA and REVA, respectively[1]. Such exposures would not have been sustained over a 24 hour period even in dry conditions, in areas which experienced substantial ashfall during even the most active phases of the Soufrière Hills eruption (Searl et al., 2002). Instead, from a hazard assessment approach, the doses used can be considered as a 'worst-case' for such an exposure to humans.

5.4.3 Biological effects following exposure to volcanic ash

The ash from the Soufrière Hills volcano has been extensively studied over the past two decades (Baxter et al., 2014) and is well characterized for its physical and chemical properties, including the sample used here (Horwell, 2007, Horwell et al., 2007, 2003a, 2003b, 2014). The biological impact of Soufrière Hills ash has also received increased attention during this time (Cullen and Searl, 1998, Cullen et al., 2002, Horwell et al., 2013, Damby et al., 2016), particularly due to the substantial crystalline silica present in ash derived from collapses of the Soufrière Hills lava dome (Horwell et al., 2014, Baxter et al., 1999).

Previous results from monoculture *in vitro* studies performed with Soufrière Hills ash are variable, due to the various and numerous different experimental designs employed and endpoints considered as well as a large amount of natural variability amongst ash samples (Horwell and Baxter, 2006, Damby et al., 2016). Despite this variability, Soufrière Hills ash is generally considered to be non-cytotoxic and to have low oxidative potential, but has the capacity to incite a low (pro-)inflammatory response (Damby et al., 2018, Damby et al., 2016, Horwell et al., 2013, Damby et al., 2013). Previous cell-specific studies on macrophages (PMA-differentiated THP1 monocytes) and epithelial type II cells (A549) indicate minimal LDH-assessed cytotoxicity and reduced GSH depletion following exposures (Damby et al., 2016), however, the response of other antigen-presenting cells to ash is largely uncharacterised. Similar findings have also been noted from *in vivo* studies

[1] Average daily tissue dose (SEVA) = 0.26 µg/ cm^2 (*i.e.*, mass per cm^2 of alveoli surface area)
Mass of deposited particles over 1 day = (0.26 µg/ cm^2 * 1,000,000 cm^2) / 1000 = 260 mg
Mass deposited over 1 day per m^3 (*i.e.*, 10 % of average airborne concentration) = 260 mg / 25 m^3 = 10.4 mg/m^3
Average daily airborne concentration (for SEVA dose) = 10.4 mg/m^3 * 10 = 104 mg/m^3

(Cullen et al., 2002, Berube et al., 2004, Housley et al., 2002, Lee and Richards, 2004).

In this study, assessment of the biological response from the triple cell co-cultures following volcanic ash exposures (both SEVA and REVA) resulted in no significant ($p > 0.05$) cytotoxicity, changes in cellular morphology, oxidative stress or release of TNF-α or IL-8. Therefore, the findings of this study are largely in congruence with previous research with Soufrière Hills ash (Cullen and Searl, 1998, Wilson et al., 2000, Damby et al., 2016).

5.4.4 Biological effects following exposure to DEP

In the present study, DEPs, alone, caused no significant ($p > 0.05$) cytotoxicity or oxidative stress to the co-culture. Previous studies also conducted with the same multicellular model have shown that DEP do not enhance the release of LDH, although it has been found that they induce oxidative stress (Clift et al., 2014b, Lehmann et al., 2009, Müller et al., 2010). Variation among studies can be perhaps attributed to differences in the applied exposure method (Holder et al., 2008, Lenz et al., 2009). In the studies referred to, above, the co-cultures were exposed to the same type of DEP (NIST 2975) but applied in suspension (in supplemented cell culture medium) to the upper chamber of the insert (Clift et al., 2014b, Müller et al., 2010), whereas, in the present study, as previously mentioned, a pseudo-ALI approach was used (Endes et al., 2014).

The lack of cytotoxicity and oxidative stress observed in the present study is contrary to previous findings using monocultures of these cell types and varying DEP type (Jantzen et al., 2012, Li et al., 2003, Bai et al., 2001, Pan et al., 2004, Xiao et al., 2003). This difference could perhaps be associated with the cellular interplay exhibited by this multicellular model, where two immune cell types (macrophages and dendritic cells) can directly interact with each other within the epithelium during reactions to particulate antigens, thus resulting in a different response (Blank et al., 2007). This is in agreement with other studies which have reported differences in biochemical responses between mono- and co-culture systems (Clift

et al., 2014a, 2011, Gasser et al., 2012). Overall, though, it should be taken into consideration that different cell cultures, DEP compositions, the preparation of particle suspension and doses used in different studies vary, which makes a direct comparison amongst these studies challenging.

Nonetheless, in the current study, DEP did cause an increased release of measured (pro-)inflammatory markers (TNF-α, IL-8 and IL-1β) compared to the negative control (supplemented cell culture medium). These findings concur with previous observations of monoculture *in vitro* studies, which reported DEP to be highly (pro)-inflammatory (Schwarze et al., 2013, Donaldson et al., 2005), as well as with studies using the same triple cell co-culture system (Clift et al., 2014b).

5.4.5 Biological effects following combined exposure to DEP and volcanic ash

At sub-lethal concentrations, as with the SEVA, REVA and DEP alone, the combined exposures (DEP+SEVA and DEP+REVA) showed no significant cytotoxicity in the triple cell co-culture. In the treatments including DEP, however, it appears that the size of nuclei and cell densities might have changed compared to the untreated cells (*Fig. 5-7*). In the experiments of the present study, cell division was assumed to be similar in all treatments and the cell number was not monitored carefully by LSM due to the insufficient representative images/samples available. Thus, it would be beneficial in future studies to include cell division/nuclear shrinkage experiments in parallel to the cell exposure experiments to try to relate possible effects of exposures to cell proliferation.

The impact of any particle type upon the respiratory system is commonly associated with an increased level of oxidative stress (Donaldson et al., 2003). Yet, in the current study, it was observed that no significant differences in oxidative stress levels were evident in any of the combined particle exposures compared to the negative control (*Fig. 5-6B*). In light of these observation, DEP, alone, showed no deviation from the negative control, but volcanic ash treatment, alone, increased the relative abundance of reduced GSH, an observation previously attributed to increased production by macrophages (in monoculture) to cope with volcanic ash (Damby et al., 2016). Therefore, comparatively, the effect of DEP on

GSH levels is greater than the effect seen with ash alone, and the effects noted with the combined exposure scenario could be attributed to the DEP driving an oxidative stress environment in the cell cultures rather than the absence of any oxidative stress. However, to elucidate the underlining mechanisms controlling oxidative stress levels in this combined exposure, further research is needed.

Despite the lack of any cytotoxic effects, and limited oxidative stress response, it was found that a heightened (pro-)inflammatory response occurred following exposure to respirable volcanic ash and DEP when applied as a combined exposure. Focussing firstly on TNF-α release, the observed effects of the combined exposures seem to be greater than the response noted for DEP or ash, independently. Furthermore, as with the GSH data, the impact of the DEP can also be seen in the combined exposure IL-8 response. However, the positive control (LPS) did not induce a significant increase in IL-8 in this particular batch of experiments, making it difficult to reliably assess the effects of tested particles.

Release of IL-1β was, however, significantly higher ($p < 0.05$) in the DEP+REVA scenario compared to the negative control and to DEP alone. Since no VA controls for this marker were included, it is difficult to assess whether there was an additive or synergistic interaction between DEP and VA. It is hypothesised here that the observed higher response of (pro-)inflammatory markers following exposure to the DEP+REVA compared to the DEP+SEVA scenario can probably be attributed to the effect of the greater combined dose of particles delivered to the cell surface, *i.e.*, additive effect. In addition,

It is not yet clear whether this effect is directly driven by the individual particle-cell interactions, secondary toxicology mechanisms incited *via* the particles' physicochemical characteristics, or through particle-particle interactions leading to the combined effect noted. Based on the present first insight into the biological reactivity of such co-exposure treatment, it is difficult to state whether the observed changes would result in an adverse acute biological impact.

Yet, it is known that increased release of (pro-)inflammatory mediators may augment, as well as prolong, inflammatory reactions and, if the exposure persists, can result in chronic inflammation (Schwarze et al., 2013). Airway inflammation not only promotes the development of lung diseases, but it may increase the susceptibility to acute cardiovascular disease (Donaldson et al., 2001). Thus, the importance of these findings lies in the potential effects on respiratory health that this combined exposure may elucidate over a prolonged period of exposure.

5.5 Conclusions

This study is the first to assess the respiratory hazard of combined exposures to urban pollution and volcanic ash. It is also the first to use a realistic, multicellular model to assess the *in vitro* toxicity of volcanic ash and to study the interactions of ash with dendritic cells.

Combined exposure to sub-lethal concentrations of an urban pollutant, *i.e.*, DEP, and respirable-sized volcanic was shown to promote a(pro-)inflammatory response *in vitro*, implying a potentially-greater hazard of simultaneously inhaling both particle types.

These initial results, especially regarding methodology, were used to inform further *in vitro* work of this thesis (***Chapter 4 and 6***). Furthermore, findings of this study were used as a base for the follow-up study investigating combined exposure to volcanic ash and a complete vehicle exhaust (***Chapter 6***).

5.6 References

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Chapter 6

An *in vitro* study of the potential respiratory hazard of combined exposure to volcanic ash and complete gasoline exhaust

Acknowledgments

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6.1 Introduction

Concerns about the potential health impacts of concomitant exposure to volcanic and anthropogenic emissions have been raised (Loughlin et al., 2012, Kar-Purkayastha et al., 2012), especially considering the known ability of particulate matter to negatively impact respiratory health (as discussed in the previous chapters, ***Chapter 2*** and ***5***). The findings of the first investigation into the potential effects of volcanic ash exposure combined with an urban pollutant, *i.e.*, exhaust particulate (***Chapter 5***) showed that concomitant exposure of cells to respirable volcanic ash and standardised diesel exhaust particles (DEP (NIST SRM 2975)) can inducerelease of (pro-)inflammatory markers *in vitro*. The understanding of the respiratory hazard which may result from these combined exposures still remains limited, especially since this first study only considered DEP and not complete exhaust (*i.e.*, including the additional gaseous component).

Exhaust emissions are a complex mixture that contains particles but, also, condensed and gaseous fractions (***Chapter 2***, *Section 2.8.1*). These phases can impact lung health (*e.g.*, Reed et al., 2008), but could also interact directly with the ash. This interaction may result in the adsorption of inorganic gases, such as CO_2, CO and NO_x, and volatile organic compounds (*e.g.,* linear and polycyclic aromatic hydrocarbons) onto volcanic ash, potentially altering the ash surface chemical properties and affecting its potential toxicity, as discussed in ***Chapter 7***. Hence, the use of complete exhaust is a critical next step in deducing the hazard posed to populations exposed to volcanic emissions and urban air pollutants.

The aim of the study in this chapter was to assess the biological impact of combined exposure to cells of respirable volcanic ash and complete vehicle exhaust. A sophisticated *in vitro* approach, as also used in ***Chapters 4*** and ***5***, provides a valuable first assessment of the potential adverse impacts of such exposures, especially due to a lack of epidemiological studies that consider health effects of ashfall in heavily polluted urban areas.

Volcanic ash samples, from Soufrière Hills volcano, Montserrat, and Chaitén volcano, Chile, were used (to represent different magmatic compositions and eruption styles; see ***Chapter 3***, *Section 3.2*) in combination with freshly-generated complete exhaust from a gasoline direct injection (GDI) vehicle (containing the particulate, condensed and gaseous fractions). This was the first time that a real, complete exhaust has been used to study combined exposures with volcanic ash. Furthermore, this investigation was the first to evaluate and report on whether the toxicity of either volcanic ash or complete gasoline exhaust are altered by co-exposures, as well as whether the ash (magmatic) composition could influence the outcome of combined exposures *in vitro*.

6.2 Methods

As with the previous studies (***Chapter 4*** and ***5***), *in vitro* experiments were carried out using a multicellular human lung model cultured at the air-liquid interface (ALI) (***Chapter 3***, *Section 3.5.1*). The dry, respirable fraction (isolated as described in ***Chapter 3***, *Section 3.3.1*) of the volcanic ash from Soufrière Hills volcano, Montserrat or Chaitén volcano, Chile (see ***Chapter 3*** *Section 3.2.1* and *3.2.2*) was nebulised over the cell cultures using a dry powder insufflator (***Chapter 3***, *Section 3.5.2.1*). Ash samples have been physicochemically characterised with regards of their particle size distribution, particle morphology and bulk chemical composition, using laser diffraction, SEM and XRF analyses, respectively (as described in ***Chapter 3,*** *Section 3.4*).

The multicellular model was co-exposed to respirable volcanic ash and gasoline exhaust, as well as to both individually, for a period of 48 hours. Details on the experimental design of cell exposures are described in *Section 6.2.3*. A sophisticated, well-characterised exhaust exposure system was employed (see *Section 6.2.1*). Following exposure, subsequent analyses of the cell cultures for common biological endpoints including cytotoxicity (lactate dehydrogenase (LDH) release and the expression of pro-apoptotic genes FAS receptor (FAS) and caspase 7

(CASP7)), oxidative stress (heme oxygenase 1 (HMOX1) and NAD(P)H dehydrogenase [quinone] 1 (NQO1) gene expression) and (pro-)inflammatory response (tumour necrosis tumour necrosis factor α (TNF-α), interleukin 8 (IL-8) and interleukin 1β (IL-1β) production at the gene and protein levels) were undertaken. The impact of exposures upon cell morphology was visualised *via* confocal laser scanning microscopy (LSM). Additional information about bioanalysis assays performed is outlined in ***Chapter 3***, *Section 3.5*.

6.2.1 Vehicle exhaust exposure system

The exposure system used in this study had been specifically developed to investigate the toxic effects of exhaust emission (Müller et al., 2011), and it had previously accompanied hazard assessment studies of products of different engines and/or fuels using similar *in vitro* epithelial airway barrier models (Bisig et al., 2018, 2016, 2015, Steiner et al., 2013a, 2013b, Müller et al., 2012, 2010).

In this study, a gasoline exhaust was used since using a diesel-fuelled vehicle was not possible during the time-frame of the experiments. A flex-fuel GDI vehicle with a three-way catalyst was driven on a chassis dynamometer with standard market gasoline (RON 95) and lubrication oil (*Fig. 6-1A*). The type and operating conditions of the engine, the compositions of the fuel and lubricating oil used, and the presence of an emission control system are factors influencing the chemical composition of a vehicle exhaust emission (Johnson, 1988). A dynamic, worldwide light-duty test cycle (WLTC) (UNECE, 2016), representing transient driving in urban, extra-urban, highway and motorway conditions, was driven and repeated for 6 h per day of exposures (10 cycles). The WLTC is the official driving cycle (from September 2017 onwards) used by the European Union for new vehicle registration (Euro6).

The exhaust exposure experiments were performed at the exhaust gas control station of the Bern University of Applied Sciences in Nidau, Switzerland, as previously described (Bisig et al., 2015, Müller et al., 2011, 2010). The exhaust was diluted 1:10 in filtered air (*Fig. 6-1A*), based on previous work (Steiner et al., 2013a, 2013b) and to enable comparison with previous gasoline exhaust studies

(Bisig et al., 2016, 2015), where it was noted that it represents a highly-polluted site (*i.e.* a high dose exposure). In addition, dilution of the exhaust ensures that the condensation of hot, emitted exhaust and alteration of the emitted particle fraction are avoided (Müller et al., 2011).

The diluted exhaust sample then entered a heated isolation box where the two exposure chambers were located (*Fig. 6-1A*). It was pumped through the cell culture exposure chamber (*Fig. 6-1B*) with a constant flow of 2 L/min. In the chamber, the exhaust emissions pass above the cell culture plates and diffuse onto the cell cultures (Müller et al., 2011). Simultaneously, in a reference chamber (*Fig. 6-1B*), filtered ambient air supplied under identical conditions served as the negative control. In order to reach the necessary conditions required by cell cultures, after the exhaust emission dilution, CO_2 was added to reach a final concentration of 5 %. The concentration was controlled with two sensors, right before and after the chamber, and adjusted with a flowmeter, if needed. The conditions in both chambers were controlled at standardised conditions for cell cultures of 37 °C, 85 % relative humidity and 5 % CO_2.

6.2.2 Exhaust characterisation

Characterisation of the exhaust was performed in parallel to the exposure experiments, yielding detailed information on the emission sample that the cells were exposed to. Measurements were taken over the duration of the exposure experiments (the initial exposures (n=2) and repeated exposures (n=2), each 10 WLTC cycles) resulting in 4 distinct datasets.

The particle number (PN) was measured in the 1:10 diluted exhaust using an engine exhaust condensation particle counter (Model 3790, *TSI Inc.*, USA) (*Fig. 6-1A*). The concentrations of carbon monoxide (CO), carbon dioxide (CO_2), total gaseous hydrocarbons (THC), non-methane hydrocarbons (NMHC) and nitrogen oxides (NO_x) were measured using a Horiba MEXA-9400H (*Horiba*, Japan) exhaust gas measuring system in a constant volume sampling tunnel (Horiba CVS-9500 T, *Horiba,* Japan).

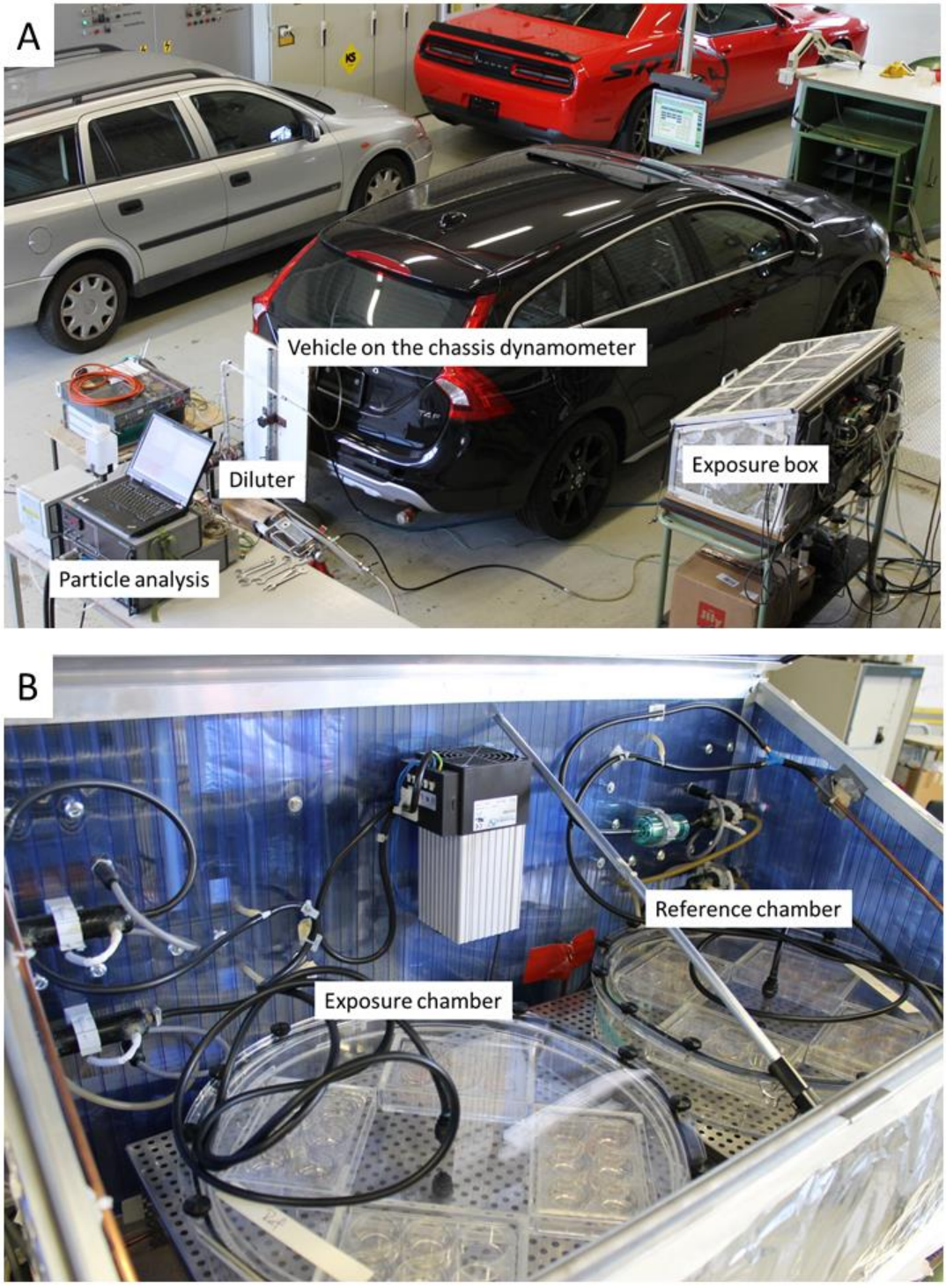

Figure 6-1: Vehicle exhaust emission exposure system. A) Overview of the exhaust emission exposure system with the vehicle used in the experiments on the chassis dynamometer, the diluter, instrumentation for the characterisation of the particulate compounds and the exposure box. **B)** The interior of the exposure box containing exposure (left) and reference (right) chambers for the exposure of cell cultures. Photos taken by the author at the exhaust gas control station of the Bern University of Applied Sciences in Nidau, Switzerland (in March 2017).

6.2.3 Cell culture exposures

The two-day cell exposure scenario (*Fig. 6-2*) was designed to simulate a real-life situation where volcanic ash is introduced to the urban environment (where people are continuously exposed to urban pollution; *e.g.,* gasoline vehicle emissions), resulting in pollutants being concomitantly inhaled.

Two sampling time-points were chosen, 24 h and 48 h, to enable observation of the effects after one day of co-exposure (as in the previous study, ***Chapter 5***) and, in addition, to account for possible effects following another, repeated exposure to gasoline exhaust.

The multicellular lung model was exposed at the ALI to diluted exhaust (1:10) or filtered air (reference chamber) continuously for 6 h (*i.e.*, the initial exposure, see *Fig. 6-3A*), followed by immediate exposure to respirable volcanic ash (*Fig. 6-3B*), and then incubated (at 37 °C and 5 % CO_2) for 18 h (*Fig. 6-2*), maintaining the ALI conditions. Subsequently, the supernatants (*i.e.*, cell culture medium) were collected (24 h time-point) from the basal side of the insert and replaced with fresh cell medium. Cells were exposed again for 6 h to diluted exhaust or filtered air (*i.e.*, the repeated exposure), followed by a final 18 h incubation, maintained under ALI conditions (see *Fig. 6-3A*). The supernatants were then collected (48 h time-point; *Fig. 6-2*).

Each 48-hour exposure scenario (initial exhaust, volcanic ash, repeated exhaust) was conducted with two different sets of the multicellular lung model (*i.e.*, with cells from different passage numbers and monocyte isolations), each exposed separately to volcanic ash after the initial exposures, resulting in two replicates (n=2) per exposure scenario. Two exposure scenarios were conducted over a 4-day period, resulting in 4 experimental replicates in total (n=4). All data are presented relative to the filtered air (reference) cultures; however, an untreated control (kept in the incubator) was also included to assess the influence of the exposure protocol on cell response. It also served as a negative control for cell cultures which were exposed to ash alone and kept in the incubator in a parallel experiment.

Collected supernatants were stored at either 4 °C or -80 °C prior to biochemical assays. Insert membranes were split and one half of each replicate's membrane was used for gene expression analysis whilst the other half was fixed and prepared for fluorescent labelling.

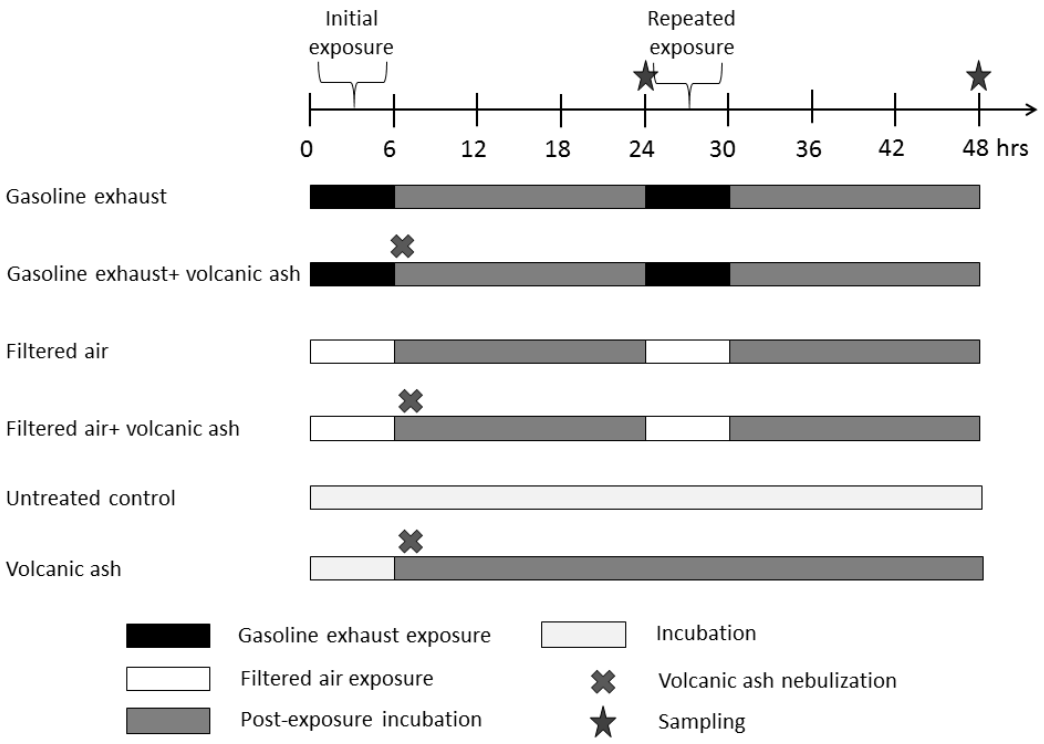

Figure 6-2: Schematic of the cell culture exposures at the ALI in the present study. Cell cultures were individually exposed to gasoline exhaust (GE) and volcanic ash (FA+VA), and then co-exposed to both (GE+VA). Here, filtered air exposure served as a (reference) negative control. Separately, additional exposures of cell cultures to volcanic ash were conducted with their respective negative control being the untreated cells (all kept in the incubator). Culture supernatant was sampled at the 24 h time-point, and both the supernatant and insert membranes were sampled at the 48 h time-point.

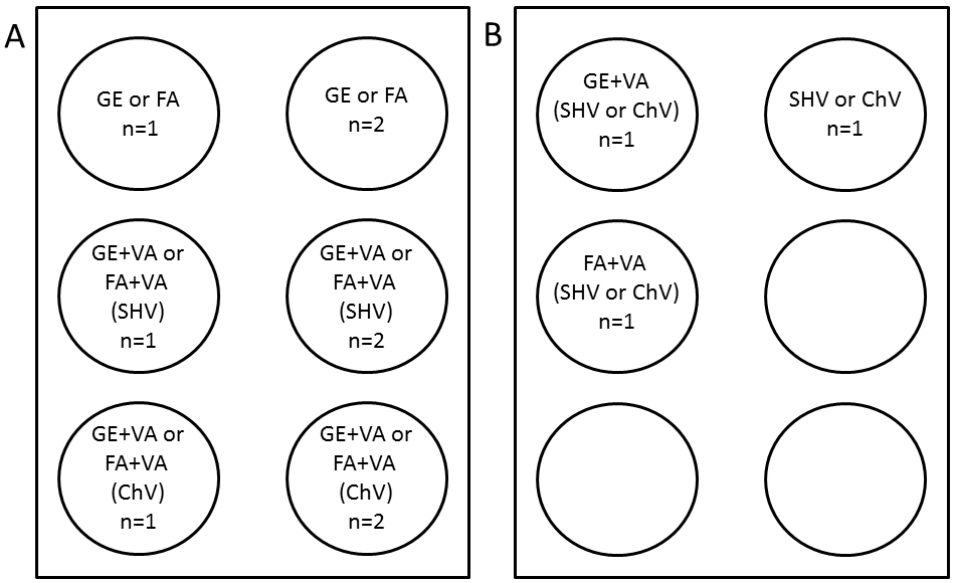

Figure 6-3: Cell culture inserts setup in the 6-well plate during the exposures. A) The exposure chamber (gasoline exhaust (GE) exposure) or reference chamber (filtered air (FA) exposure) insert setup during initial (6 h, day 1) and repeated exposures (6 h, day 2), simultaneously exposing two replicates (n=2). **B)** Volcanic ash exposures insert setup which followed initial gasoline exhaust exposures, exposing one replicate for each scenario (co-exposure (GE+VA), post-reference chamber ash exposure (FA+VA), volcanic ash exposure (SHV or ChV)) at the time (n=1).

6.2.3.1 Exposure doses

Daily experimental exposure to diluted (1:10) gasoline exhaust for 6 h was chosen according to earlier studies (Bisig et al., 2016, 2015, Steiner et al., 2013a, 2012). It is difficult to assess whether this represents real life exposures, but could represent exposure of an urban outdoor worker.

The cell-delivered volcanic ash doses fall within the range of the lowest and highest doses used in the previous study (***Chapter 5***). In order to achieve equivalent mass exposure for both ash samples, the QCM was monitored during the exposures until a target dose between 0.4 and 0.5 µg/cm^2 was reached. There is a lack of dosimetry data for inhalation of ash, or exposure data on ambient air concentrations following volcanic eruptions, making average exposures difficult to constrain and apply *in vitro* (see ***Chapter 2***, *Section 2.5.1*), but in the previous study (***Chapter 5***) it was determined this dose range to be a worst-case scenario. Therefore, these doses may not be realistic for personal exposure and could be considered as a particle over-load relative to a real-life exposure.

Overall, the chosen exposure scenario may be considered as a short-term, high-level exposure to both pollutants, individually and when combined. Even though the doses used likely deviate from realistic inhalation exposure, the assessment of cellular responses herein can be seen as a valuable screening of possible (adverse) effects that this specific type of concomitant exposure may incite which has not previously been considered or investigated.

6.3 Results

6.3.1 Volcanic ash characterisation

Particle size analysis of isolated respirable fractions showed that 98 % by volume and 84 % by volume of particles were sub-10 µm diameter, for ash from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV), respectively (*Fig. 6-4A*). The SHV sample consisted of 58 % by volume particles with size < 4 µm, while ChV contained less with 40 % by volume.

The morphology of the particles from both volcanoes, as observed by SEM, was mostly blocky and angular with varying amounts of sub-micron particles adhering to the surfaces of larger particles (*Fig. 6-4B*). This is congruent with previous observations of respirable volcanic ash (*e.g.*, Damby et al., 2016, Lahde et al., 2013, Horwell et al., 2013, Hillman et al., 2012, Le Blond et al., 2010), but may not mirror the morphology of larger ash particles, which can differ according to (magmatic) composition.

Bulk oxide elemental data for samples are listed in *Table 6-1* and indicate magmatic composition of the ash samples. The SHV ash was confirmed to be 'andesitic', with an intermediate composition regarding silicon dioxide (SiO_2) content (61.77 wt. % SiO_2), while ChV is 'rhyolitic', being comparatively richer in SiO_2 (73.42 wt. % SiO_2). Another notable difference between the chosen samples is the crystalline silica content; the SHV ash is rich in crystalline silica (~12 wt. % of the

bulk ash is cristobalite), whereas, in comparison, ChV contains substantially less crystalline silica (~3 wt. % of the bulk ash is cristobalite) (Horwell, 2007, 2010).

Table 6-1: Bulk chemical compositions of the volcanic ash samples used in the study. Results are presented as component weight percent oxide and recalculated to include loss on ignition (LOI) in the final total.

Sample	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	SO_3	LOI	Total
SHV	61.8	0.5	17.0	6.6	0.2	2.4	6.3	3.7	0.9	0.1	0.1	0.6	100.1
ChV	73.4	0.2	13.9	1.6	0.1	0.4	1.5	4.2	2.9	0.1	0.0	1.1	99.4

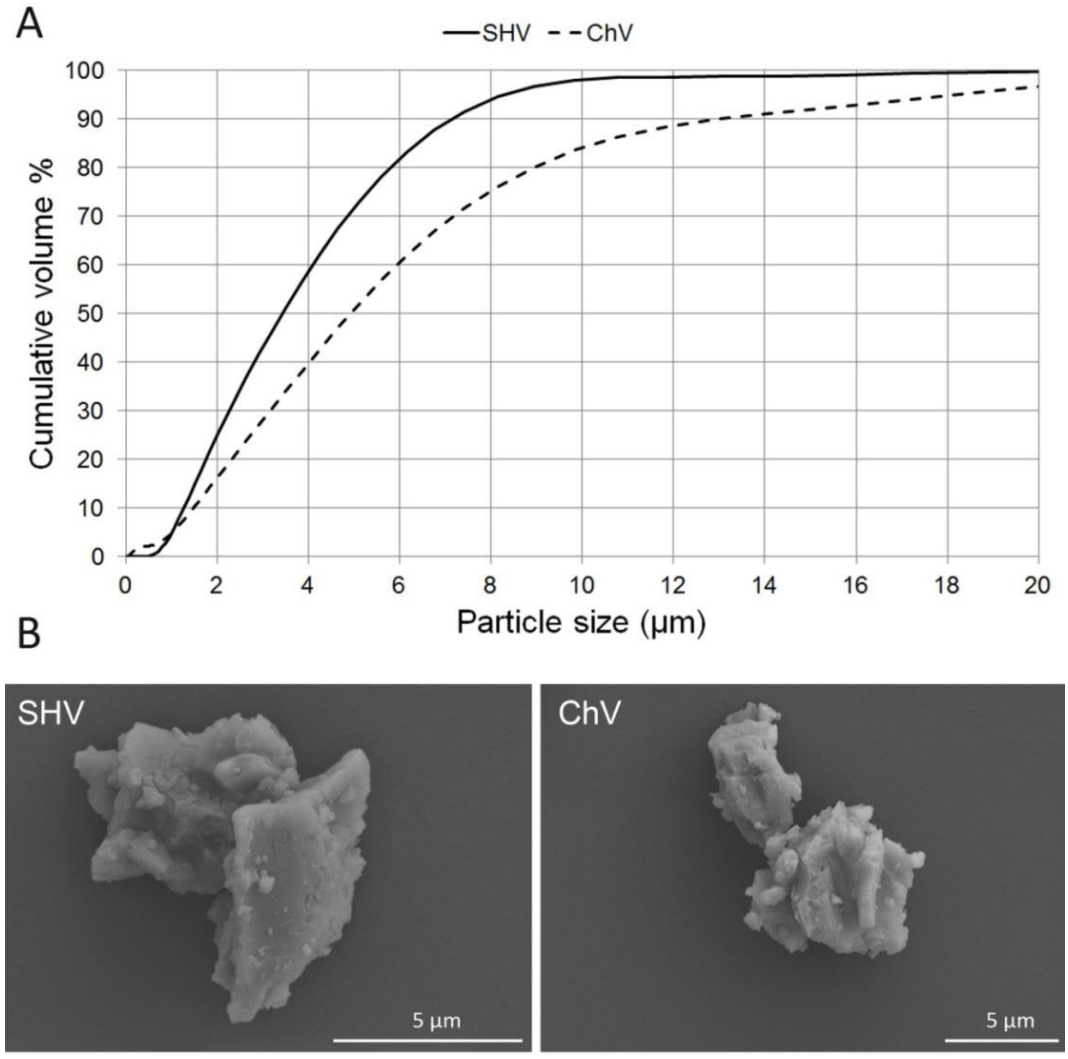

Figure 6-4: Volcanic ash characterisation. A) Particle size distribution (PSD) of the isolated respirable fraction of volcanic ash samples determined by a Beckman Coulter LS 13 320 PSD analyser (*Coulter Corporation*, USA). Data are the mean of n = 3. **B)** Representative scanning electron microscopy images of volcanic ash samples from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV). Images were collected at 10.0 kV and WD 16 mm.

6.3.2 Volcanic ash nebulisation

The average cell-delivered doses of nebulized ash using the dry powder insufflator, as monitored by a QCM, were 0.54 ± 0.19 µg/cm^2 and 0.39 ± 0.09 µg/cm^2 for SHV and ChV ash, respectively (*Fig. 6-5*).

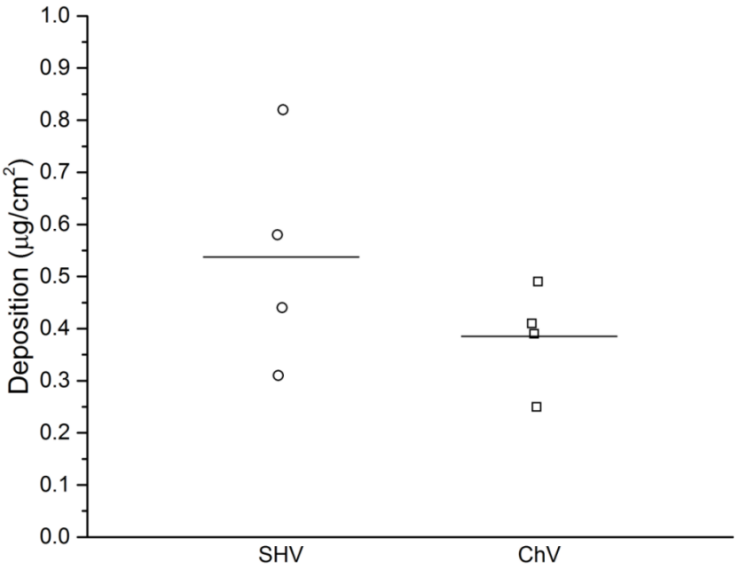

Figure 6-5: Deposition of nebulized respirable volcanic ash. Average mass deposition (µg/cm^2) of respirable ash from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV) quantified using a QCM, following their dry nebulisation over cell cultures in all employed scenarios (GE+VA, FA+VA and VA) using a dry powder insufflator (DP-4, *Penn Century*, USA). All data are presented as single values and mean (a solid line), n=4.

6.3.3 Exhaust characterisation

The composition of the gaseous fraction, comprising carbon monoxide (CO), total hydrocarbons (THC), non-methane hydrocarbons (NMHC), nitrogen oxides (NO_x) and carbon dioxide (CO_2), as well as the average count of produced particles are shown in *Table 6-2*.

Table 6-2: Average exhaust composition for the flex-fuel GDI vehicle in the WLTC as measured during the experiments (n=4). SD = standard deviation, CO = carbon monoxide, THC = total hydrocarbons, NMHC = non-methane hydrocarbons, NOx = nitrogen oxides, CO_2 = carbon dioxide. *Note that the CO_2 concentration applied to the cell culture chamber was adjusted as necessary to 5% CO_2. PN = particle number. All data are shown 1:10 diluted (as applied to the cell cultures).

Exhaust component	Unit	Mean	SD
CO	ppm	27.71	2.98
THC	ppm	6.97	0.44
NMHC	ppm	4.82	0.46
NO_x	ppm	1.76	0.12
CO_2*	%	0.98	0.01
PN	#/cm^3	1.32E+05	1.65E+04

6.3.4 Biological endpoints

For the assessed biological endpoints (cytotoxicity, oxidative stress and (pro-)inflammatory mediators, including measurements for both protein production and gene expression), no significant ($p > 0.05$) changes in cell cultures were observed at 24 h or 48 h time-points for any of the experimental exposures, *i.e.*, volcanic ash (VA; following exposure to filtered air), gasoline exhaust (GE) and co-exposures (GE+VA). In comparison to the cells treated with filtered air, the response of the untreated cells (*i.e.*, incubator control) was lower (*Fig. 6-6*), albeit not significantly.

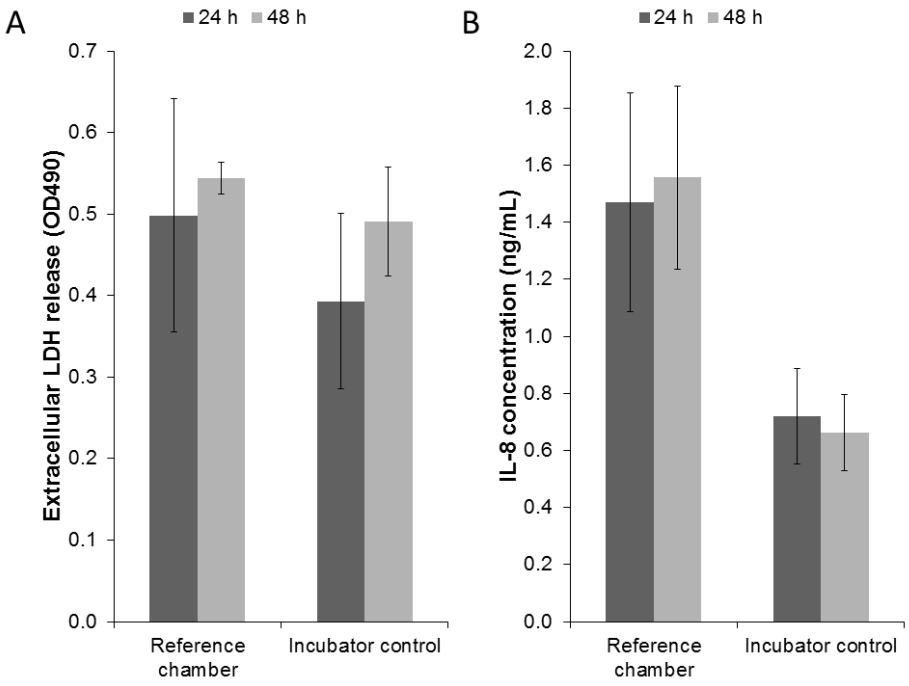

Figure 6-6: Comparison of the multicellular lung model responses for the filtered air (reference) exposure and untreated (incubator) control. A) Lactate dehydrogenase (LDH) and **B)** interleukin-8 (IL-8) release in the culture medium after 24 h and 48 h. The reference chamber culture was exposed to filtered air and incubated (37 °C, 5 % CO_2) as detailed in the main text (***Section 6.2.3***). The untreated (incubator) control was kept in the incubator (37 °C, 5 % CO_2) throughout the entire experiment, with the supernatant collected (at 24 h and 48 h) and analysed identically. Both cultures were maintained at the ALI throughout the 48-hour experiment.

Similarly, in cell cultures kept in the incubator following exposures to ash alone (SHVi and ChVi), neither of the ash samples caused a significant response in the endpoints measured (LDH and IL-8 release), when compared to the untreated cells (*Fig. 6-7*).

In the following text, the effects of ash exposures (VA) refer to the cells treated with filtered air as the comparison of the effects of cell treatments is more appropriate if made with the reference exposure chamber control, since the cells were kept in similar conditions.

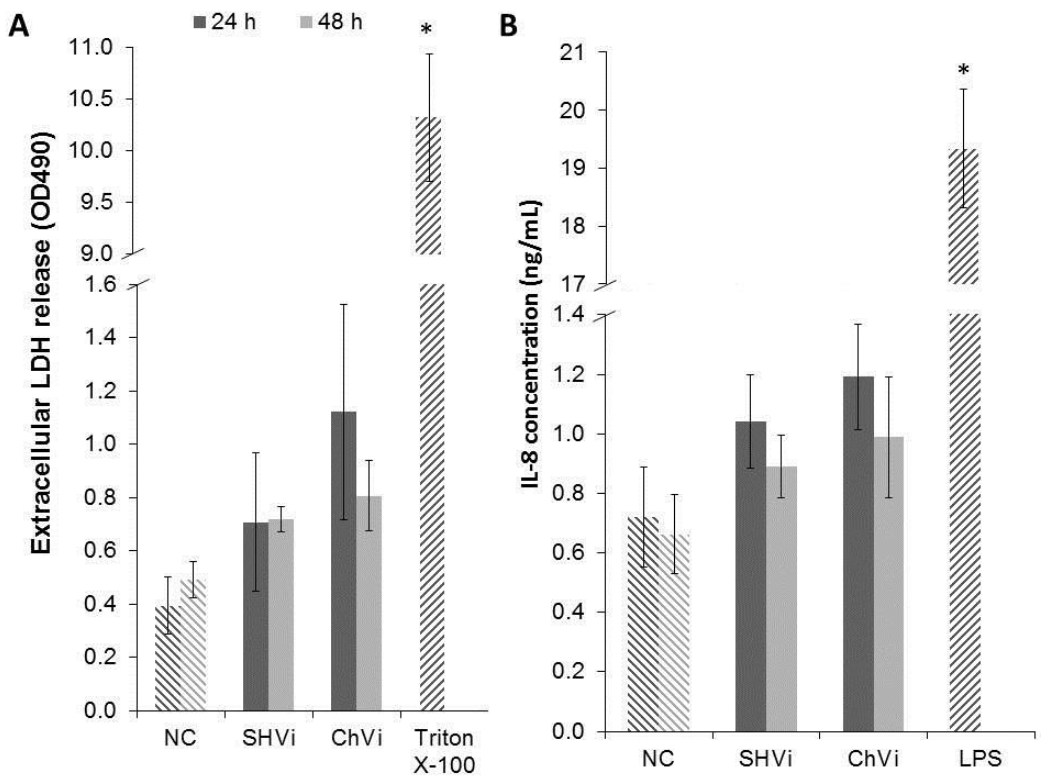

Figure 6-7: The multicellular lung model responses for the ash alone exposures. A) Lactate dehydrogenase (LDH) and **B)** interleukin-8 (IL-8) release in the culture medium following exposures to Soufrière Hills volcano (SHV) and Chaitén volcano (ChV) ash, after 24 h and 48 h. The positive assay controls were 0.2 % Triton X-100 in PBS and lipopolysaccharide (LPS; 1 µg/mL, 24 h) for LDH and IL-8, respectively. The negative control (NC) was the cell culture medium only. The cultures were maintained at the ALI and kept in the incubator (37 °C, 5 % CO_2) throughout the entire experiment (which is denoted by the subscripted 'i' in the ash sample labels). * denotes a significant difference ($p < 0.05$) between the positive control and the other samples tested.

To account for the influence of the potentially stress-inducing airflow as well as for the different baseline levels in the various cultures, the comparison of the effects of cell treatments (VA, GE and GE+VA) was made with the filtered air (reference) exposure.

For all exposure scenarios (VA, GE and GE+VA) at 48 h, LSM imaging revealed a homogenous and confluent epithelial cell layer with no alteration in cell morphology compared to the filtered air (reference) exposure (*Fig. 6-8*). Although not verified, it is possible that there could be an increase in cell number and a decreased nuclei size following treatments including GE (see discussion in ***Chapter 5***, *Section 5.4.5* for DEP treatment). LDH release by the cells following the

exposures, for both time-points, showed limited elevation in comparison to the filtered air (*Fig. 6-9A*). The positive LDH assay control Triton X-100 showed a significant ($p < 0.05$) increase in LDH content in culture medium, confirming that the biological model used was responsive for the measured endpoint. For expression of pro-apoptotic genes *FAS* and *CASP7*, none of the exposures showed a statistically significant ($p > 0.05$) outcome relative to filtered air (*Fig. 6-9B*).

As determined via release of specifically chosen (pro-)inflammatory mediators, none of the cell exposures induced a significant ($p > 0.05$) (pro-)inflammatory response (*Fig. 6-10A*). In fact, the concentrations of TNF-α and IL-1β in all measured samples were below the method detection limits (MDL; *Fig. 6-11*). LPS, which served as a positive assay control, significantly ($p < 0.05$) increased the release of IL-8 (*Fig. 6-10A*) as well as TNF-α and IL-1β (*Fig. 6-11*) compared to the filtered air and other cell treatments. Lack of a (pro-)inflammatory response was supported by the findings on a gene level, where the cell exposures did not induce any change in mRNA levels of measured markers, *IL8* and *IL1B*, relative to filtered air (*Fig. 6-10B*). In agreement with the protein measurements, none of the treatments induced a detectable upregulation of *TNFA*.

Similarly, the expression of investigated genes related to oxidative stress, namely *HMOX1* and *NQO1*, showed no significant ($p > 0.05$) increase after exposure to VA, GE or GE+VA (*Fig. 6-12*).

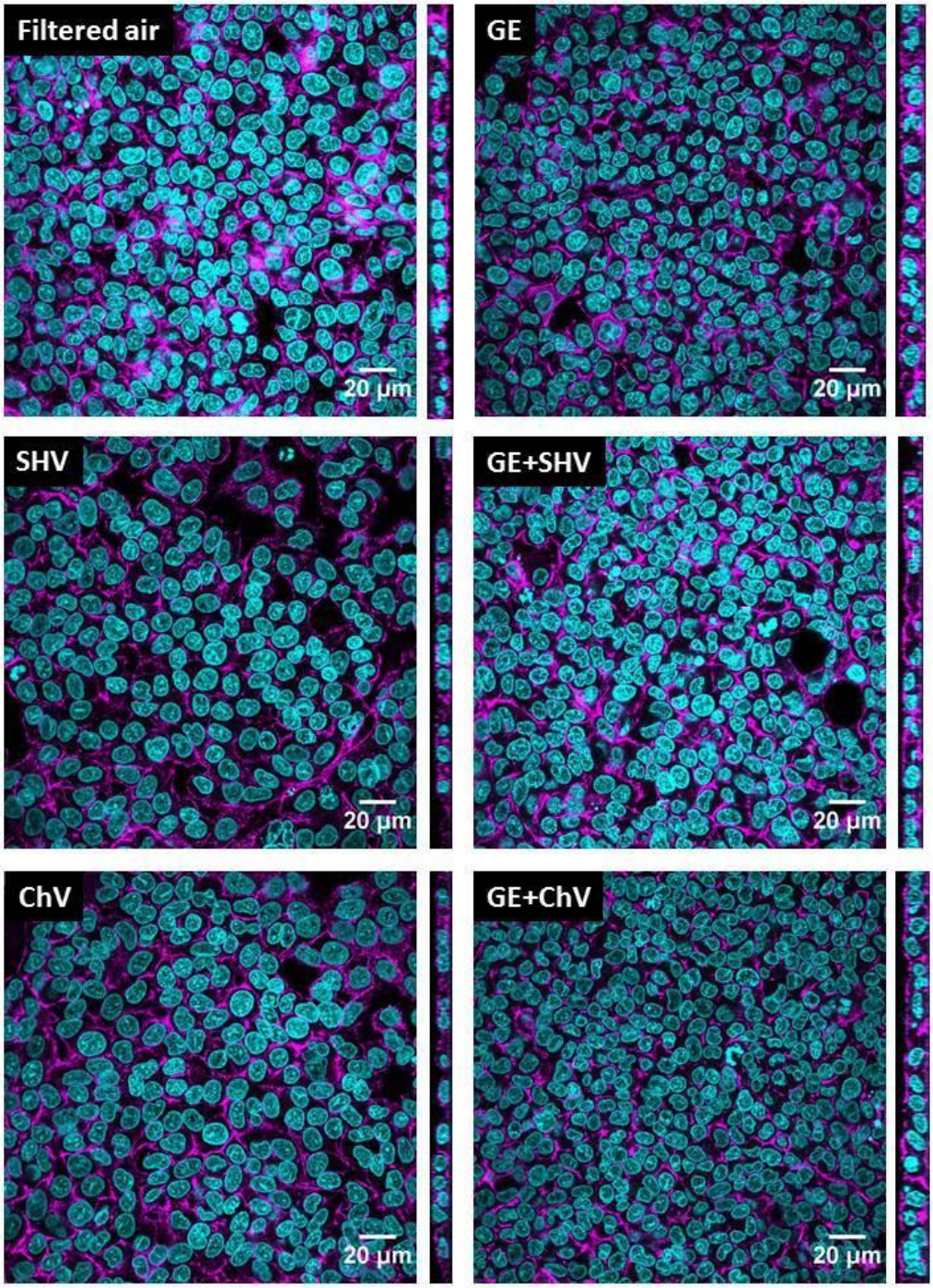

Figure 6-8: Cell morphology of the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. Representative confocal LSM images from XY and YZ projections for cultures exposed to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE+SHV), and combined exposure to gasoline exhaust and Chaitén ash (GE+ChV). Cells were stained with Phalloidin-Rhodamine (F-actin cytoskeleton, magenta) and DAPI (cell nuclei, cyan). Scale bars are 20 µm. Images were collected at x63 magnification.

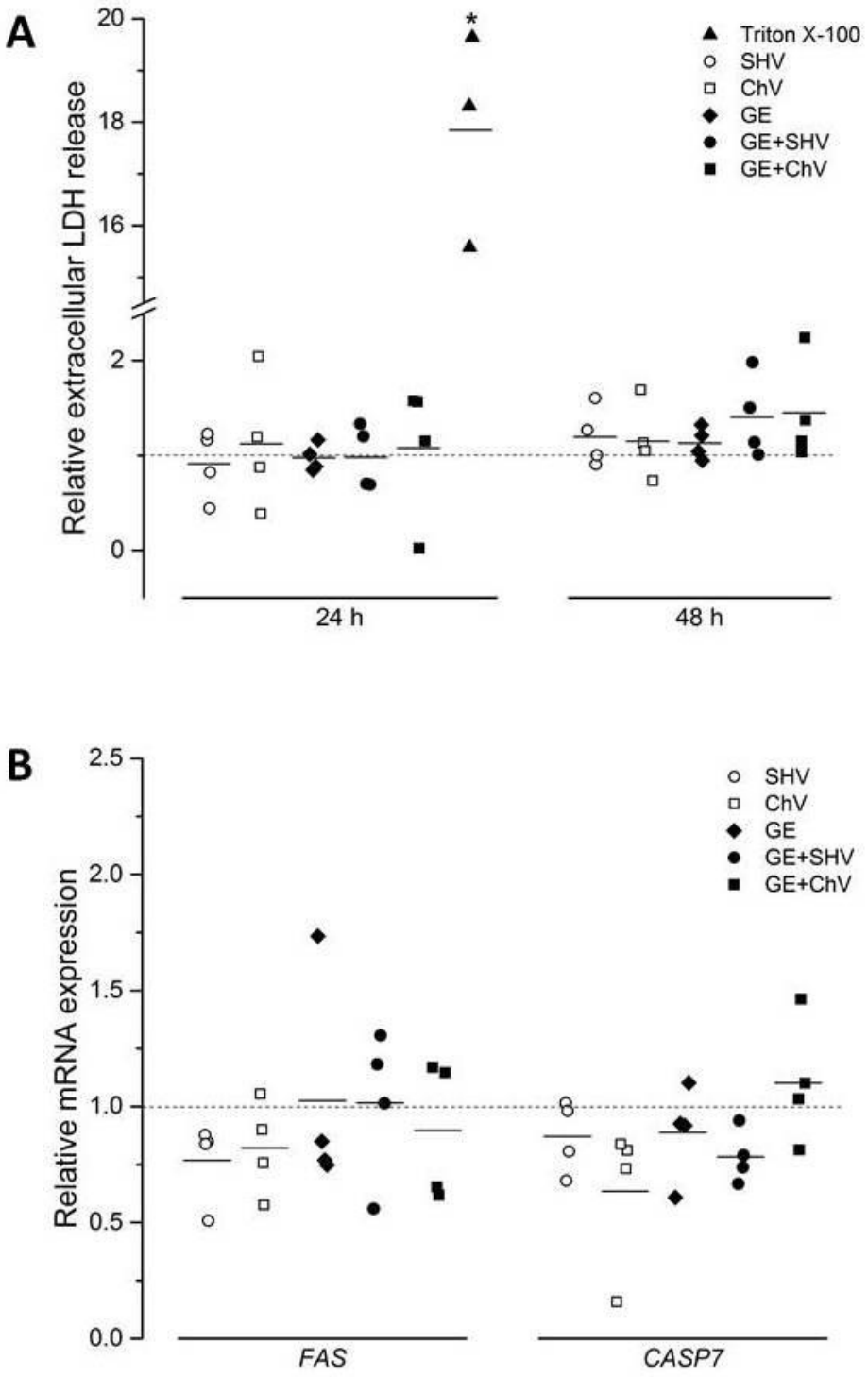

Figure 6-9: Cell viability of the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. A) Extracellular LDH levels in the culture medium after 24 h and 48 h normalized to filtered air (reference) exposure (a dashed line). The positive assay control was 0.2 % Triton X-100 in PBS. **B)** Amounts of mRNA of pro-apoptotic genes FAS receptor (FAS) and caspase 7 (CASP7), 48 h post-exposures, normalized to filtered air (reference) exposure (a dashed line). All data are presented as single values and mean (a solid line), n=4; * denotes a significant difference ($p < 0.05$) between the positive control and the other samples tested.

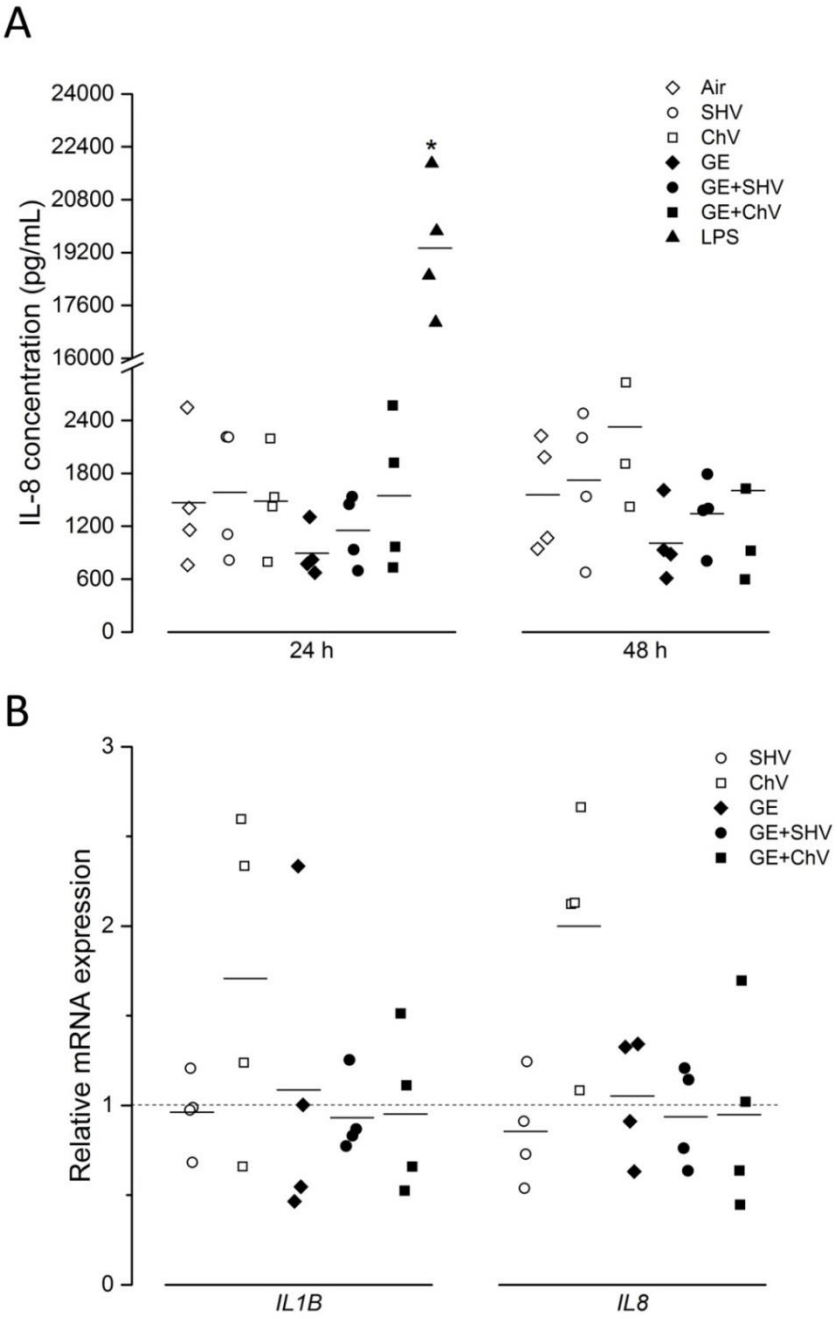

Figure 6-10: Release of (pro-)inflammatory mediators in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. A) Interleukin-8 (IL-8) release in the culture medium after 24 h and 48 h following exposures to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE+SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE+ChV). The positive assay control was lipopolysaccharide (LPS; 1 µg/mL, 24 h). **B)** Amounts of mRNA of (pro-)inflammation-related genes encoding interleukin-1 beta (IL1B) and IL-8 (IL8), 48 h post-exposures, normalized to filtered air exposure (a dashed line). All data are shown as single values and mean (a solid line), n=4; * denotes a significant difference ($p < 0.05$) between the positive control and the other samples tested.

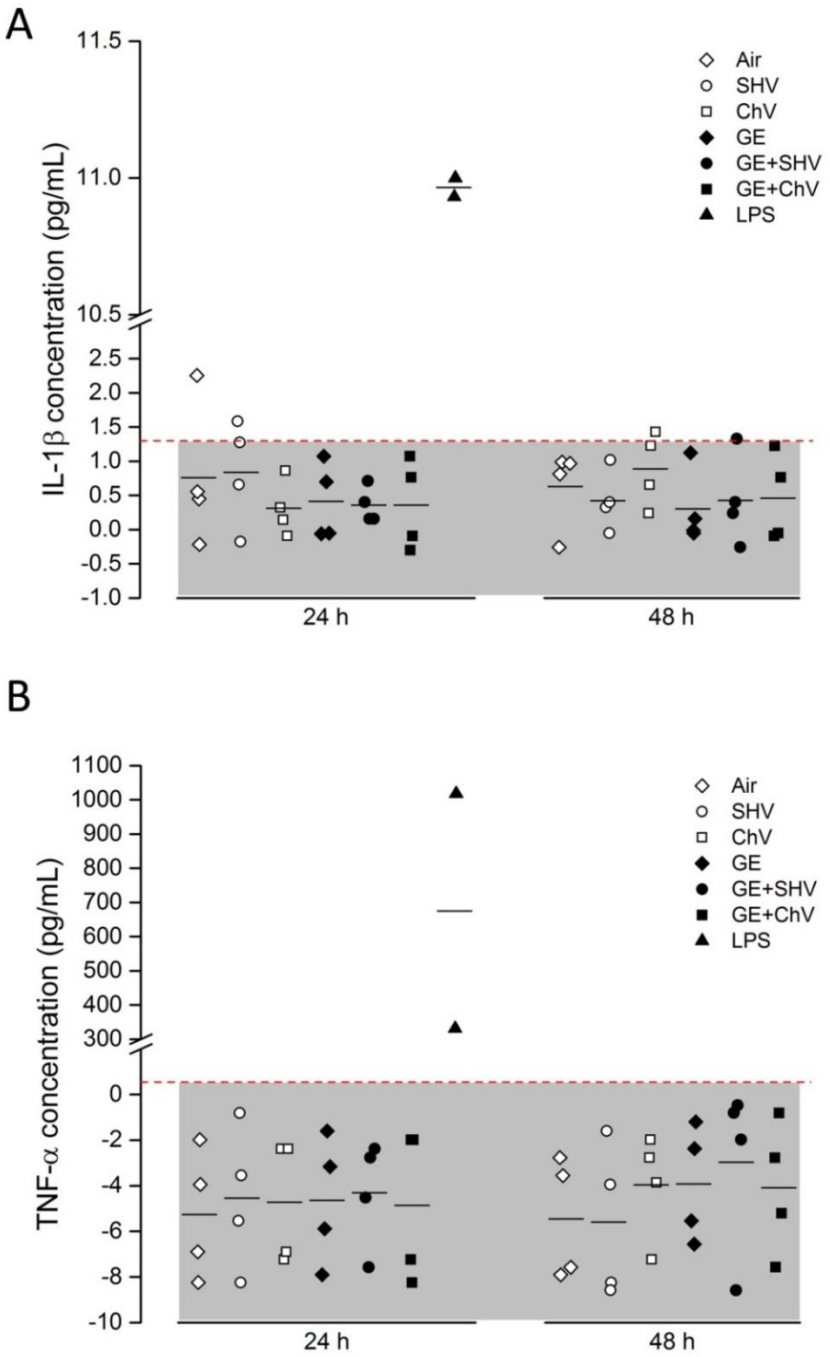

Figure 6-11: Release of (pro-)inflammatory mediators in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. A) Interleukin-1 beta (IL-1β) and **B)** Tumor necrosis factor-alpha (TNF-α) release in the culture medium after 24 h and 48 h following exposures to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE+SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE+ChV). The positive assay control was lipopolysaccharide (LPS; 1 µg/mL, 24 h). The red dashed line denotes the method detection limit (MDL; 1.30 and 0.55 pg/mL for IL-1β and TNF-α, respectively). The grey background covers the data below the MDL which may not be considered reliable and are not used in data interpretation.

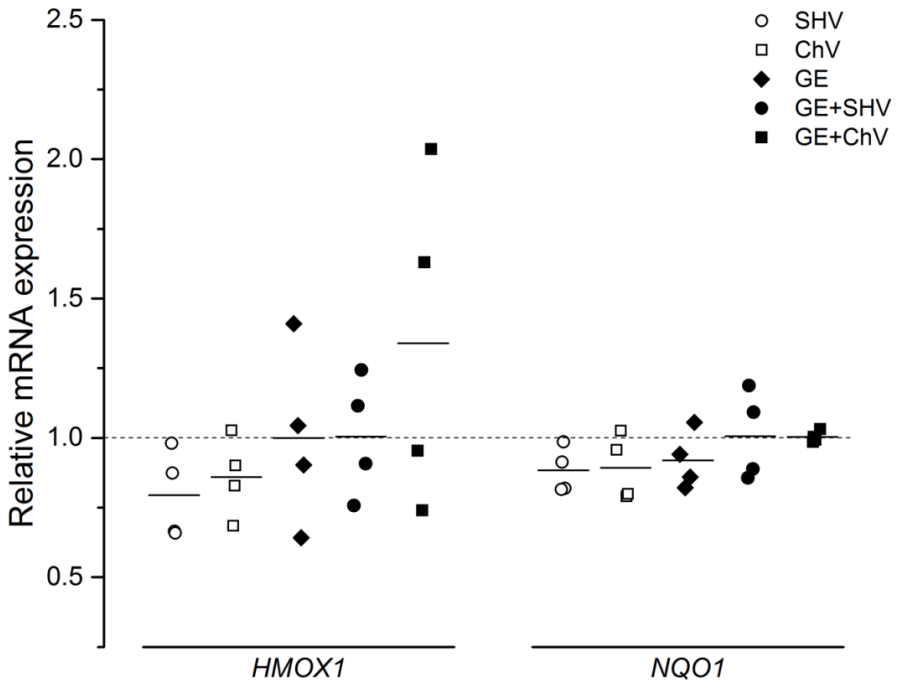

Figure 6-12: Oxidative stress response in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. Amounts of mRNA of oxidative stress responsive genes, heme oxygenase 1 (HMOX1) and NAD(P)H dehydrogenase [quinone] 1 (NQO1), following exposures to (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE+SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE+ChV), normalized to filtered air exposure (a dashed line). Data are shown as single values and mean (a solid line), n=4.

6.4 Discussion

The purpose of the study was to investigate the potential respiratory hazard of combined exposure to volcanic ash and anthropogenic pollution, through experiments designed to assess the impact to a multicellular lung model of exposure to both volcanic ash and complete gasoline exhaust.

It was found that exposure of cells to gasoline exhaust, alone, does not induce any significant effects on any of the biological endpoints measured. These results are in agreement with research performed by Bisig et al. (2016) using gasoline exhaust, alone, on a multicellular human lung model mimicking the bronchial compartment, under similar experimental conditions. Another study by Bisig et al. (2015), using the same experimental setup but a different car, found that gasoline exhaust induced oxidative stress; however, the particle number measured in the

diluted exhaust was up to three orders of magnitude higher than that used by Bisig et al. (2016), and nearly twice as high as the average daily number of particles produced during exposures in the present study (*Table 6-2*). Similarly, the concentrations of volatile compounds in Bisig et al. (2015) exceed those measured in Bisig et al. (2016) and the current study. Bisig et al. (2015) found that filtration of the particulate fraction from the exhaust was not sufficient to eliminate the adverse effects *in vitro*, confirming the importance of the volatile compounds in GE-induced toxicity. The toxic effects of gasoline exhaust, particularly after particle filtration, have also been observed with *in vivo* animal studies (Reed et al., 2008, Lund et al., 2007). It was noted, though, that volatile compounds, alone, might react differently with the lung cells than when part of the complete exhaust, where these compounds can adsorb onto the particle surfaces (Steiner et al., 2016, 2014).

The biological sensitivity of the employed multicellular model has been validated in the past through use of positive particulate controls, *e.g.*, crystalline quartz (DQ12) for a (pro-)inflammatory response (Endes et al., 2014, Chortarea et al., 2015). The lack of adverse effects observed following gasoline exhaust exposure in the present study may be explained by important differences in experimental parameters, including the employed cell lines, the driving test cycle and vehicle tested and, hence, the lower particle numbers and volatile concentrations (and consequent lower doses) as compared to other studies, and exposure times. In addition, it is known that the response of cultured cells to exposure may vary, especially over time (Poland et al., 2014). The evidence from other studies such as Bisig et al. (2015) suggests that a conclusion that gasoline exhaust is incapable of inciting a biological response should not be drawn.

The two ash types used in this study, erupted from different volcanoes of different magmatic compositions, also did not elicit a significant response in the biological endpoints measured from the multicellular model. Ash samples from these volcanoes have been previously investigated for their toxic potential and also showed limited biological responses (Damby et al., 2016, Horwell et al., 2013, Wilson et al., 2000, Cullen and Searl, 1998 and unpublished data for ChV). The SHV ash has also caused minimal response to the same multicellular model in the study

described in ***Chapter 5***. Hence, these data confirm the generally-observed lack of potential of ash to significantly affect healthy lung cell integrity or function including, in this case, initiation of an inflammatory response for the chosen time-points and endpoints.

Given the lack of significant response to gasoline exhaust, alone, and volcanic ash, alone, the finding that the co-exposures did not cause significant adverse effects in the multicellular model is perhaps not surprising, and could indicate that these combined exposures did not generate either an additive or synergistic response. Due to limitations in the experimental design (not being allowed to mobilise ash in the chamber, or mix it with exhaust prior to it reaching the chamber), the effect of direct ash-volatile interactions (*e.g.,* volatile adsorption) prior to co-culture exposures was unable to be tested here.

The absence of alterations in cell morphology, cell viability and oxidative stress state for any combined exposure scenario are in line with the study in ***Chapter 5***, which showed limited cytotoxic and oxidative potential of SHV ash when exposed concomitantly with DEP. However, the low (pro-)inflammatory response following combined exposures is contrary to the previous findings, where co-exposures of SHV ash and DEP increased release of (pro-)inflammatory mediators TNF-α and IL-8, as well as significantly increased ($p < 0.05$) IL-1β (***Chapter 5***). In ***Chapter 5***, it was hypothesized that the observed IL-8 production was driven by DEP, and that volcanic ash, alone, did not result in production of TNF-α, although it augmented TNF-α production in the co-exposures. Together with the results from Bisig et al. (2016), who report no upregulation of *TNFA* or *IL8* in response to gasoline exhaust exposure in a bronchial epithelium model, it appears that co-exposures to volcanic ash and gasoline exhaust do not induce a (pro-)inflammatory response via these pathways, at least at the doses tested here.

The similar biological responses to both ash samples in combined exposures with gasoline exhaust indicates that, within the parameters of this particular experimental setup, differences in sample composition (*e.g.,* silicon dioxide content) and mineralogy (*e.g.*, crystalline silica content) did not affect the biological

response to co-exposures. Whilst two fairly different ash samples were chosen, volcanic ash is a heterogeneous dust, the physicochemical characteristics of which can vary considerably, even during a discrete eruption (Damby et al., 2017, Horwell et al., 2013), and samples from different eruptions have shown differences in toxicity when tested comparatively *in vitro*, previously (Damby et al., 2016, Horwell et al., 2013, Wilson et al., 2000). Hence, a different sample from an individual eruption or a different ash type might incite a different cellular response.

The potential for diesel exhaust, and DEP in particular, to cause adverse respiratory effects is well known (see review by Steiner et al., 2016) while, on the contrary, the toxicity of exhaust from GDI vehicles is still relatively unknown (CCEM, 2016, Muñoz et al., 2016). Given this, it is evident that there is a need to conduct further studies to clarify the hazard posed by combined exposures, particularly with a fuel that generates exhaust of likely greater toxicity (*e.g.,* diesel) (Bisig et al., 2016), which was not possible during the time-frame of the current experiments. Future studies that consider the very complex and variable components of ambient urban air would be prudent, as would additional endpoints, such as genotoxicity, that help to derive a more comprehensive understanding of the potential hazard. Furthermore, the experimental approach in this study, although performed over a two-day period (as opposed to the commonly-used time-point of 24 h), still represents a short-term exposure scenario. Hence, potential chronic effects that such exposures could elucidate, over a prolonged period, have not been accounted for and need to be investigated.

6.5 Conclusion

This study provides the first insights into the biological effects caused by exposure to complete gasoline exhaust in the presence or absence of volcanic ash, as conducted through a realistic *in vitro* hazard assessment. The findings show that combined, and individual, gasoline exhaust and volcanic ash exposure at the ALI has limited adverse biological impact to a multicellular lung model *in vitro*, considering

the employed experimental conditions and biological endpoints measured (cytotoxicity, oxidative stress and (pro-)inflammatory response at the protein and gene levels). However, it should be considered that a different ash sample from an individual eruption or ash of a different magmatic composition, individually or co-exposed with an exhaust generated by a different type of an engine, fuel or lubrication oil, might incite a different cellular response.

More detailed investigation of the potential respiratory hazard following such combined exposures in future eruptive events is necessary, especially considering the complexity of the ambient urban air. Additional biological markers should be studied in further experiments *in vitro* and a complete diesel exhaust could also be used.

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Chapter 7

Interaction of volcanic ash with vehicle combustion products

Acknowledgments

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7.1 Introduction

It is well known that solid airborne particles can adsorb compounds from the ambient air (Usher et al., 2003b) such as polycyclic aromatic hydrocarbons (PAHs) and, thus, act as carriers of potentially toxic compounds into the lungs, where they can cause damage to lung cells and tissues (Fubini and Arean, 1999). Previous studies have demonstrated the potential of ash to scavenge volcanic gas species as well as anthropogenic volatile organic compounds (VOCs), as discussed earlier in this thesis (***Chapter 2***, *Section 2.7*). Notably, it has been shown that volcanic ash can scavenge PAHs of anthropogenic origin (Stracquadanio et al., 2003), which are known for their genotoxic potential (IARC, 1983, Long, 2017 and references therein).

A core aspect of this pilot study is the hypothesis that the interaction of volcanic ash with vehicle exhaust emissions will result in uptake of compounds by particle surfaces, potentially creating new surface functional groups which are not usually present on the ash surface. This leads to the assumption that the biological response to such ash may be different to the one observed after exposure to pristine ash erupted in an unpolluted atmosphere.

No differences in observed toxic responses could be deduced following combined exposure to volcanic ash and gasoline exhaust (***Chapter 6***), possibly due to the experimental setup, where it was not possible to mix ash with exhaust, *i.e.*, simulate the interaction of particles and gases, prior to the cellular exposures. Therefore, this chapter expands the investigation to obtain a first insight into the potential for such chemical interactions.

Generalized schemes for different heterogeneous surface reaction mechanisms that can occur during uptake of gases onto mineral dust, detailed in a review by Usher et al. (2003b) and briefly discussed here, are shown in *Fig. 7-1*. Gases can be taken up by particle surface (on reactive surface sites) in a non-reactive reversible way (*Fig. 7-1A*), which implies that the adsorbed gas may be released from the surface during particle transport. This is believed to be a common mechanism of adsorption for semi-VOCs (*e.g.*, PAHs). *Fig. 7-1B* shows the

reversible adsorption of gas which is followed by surface reaction and potentially formation of gas-phase products (*e.g.*, Ammann et al., 2003), while a similar process, where a reaction product is permanently absorbed into the bulk of the particle, is shown on *Fig. 7-1C*. If the surface of a particle is coated with a deliquescent film, gas will adsorb and interact with the film, potentially forming a gaseous product (*Fig. 7-1D*). *Fig. 7-1E* represents a mechanism of continuous gas uptake by catalytic reactive surface adsorption (*e.g.*, Usher et al., 2002, 2003a). Finally, *Fig. 7-1F* shows a complex multilayer coating of the surface and occurrence of synergistic adsorption (when an adsorbed molecule provides a reactive site for another molecule).

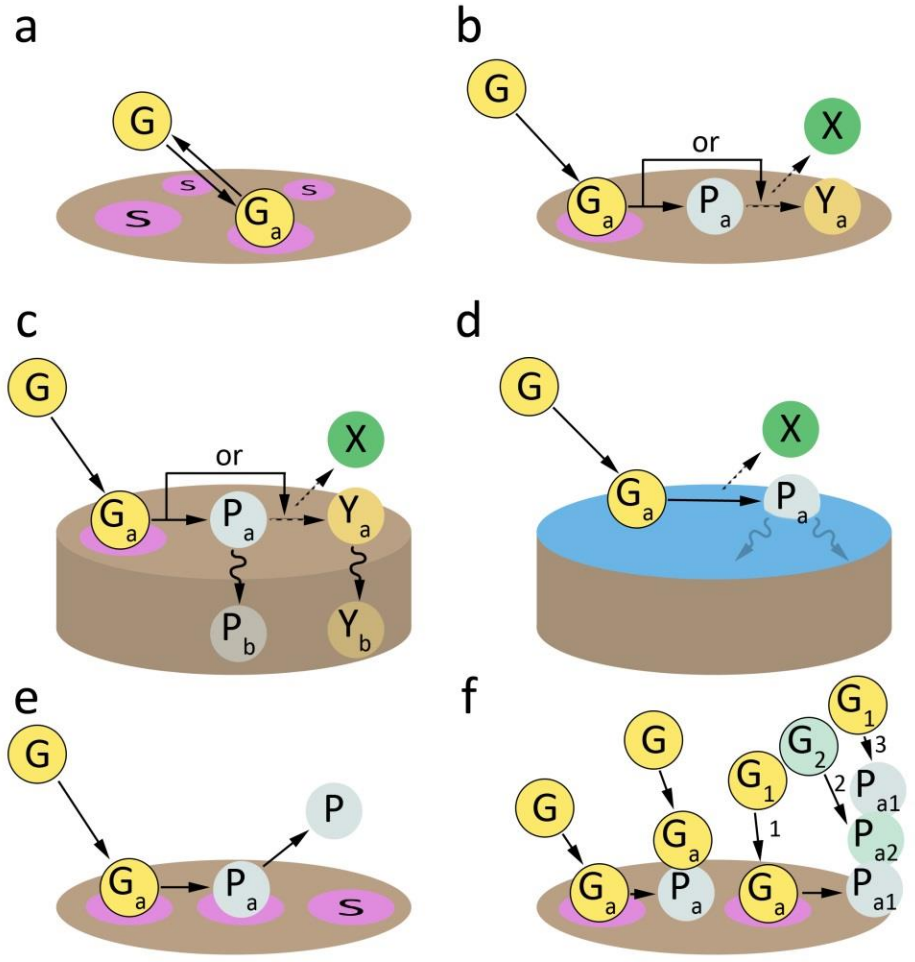

Figure 7-1: Generalized scheme for different surface reaction mechanisms of gas uptake on mineral dust (adapted from Usher et al. (2003b), produced by P-Y. Tournigand). Chemical components include surface sites (S), gas reactants (G, and product species (P, X and Y), which can be released into the gas phase, adsorbed to the surface ($_a$), and/or incorporated into the bulk ($_b$). Numbers next to arrows represent sequential reactions, while subscripted numbers signify different reactant gases.

The aim of this pilot study was to investigate, for the first time, the interactions and capacity of ash to uptake urban pollution compounds by exposing ash to synthetic exhaust, in order to understand the chemistry occurring at the particle surface. To achieve this, it was explored whether industry standard exhaust test beds can be used to understand ash-exhaust interactions. The species that may exist on the ash surface following exhaust exposure were probed with pyrolysis gas chromatography-mass spectrometry (Py-GCMS), a sensitive technique often used by organic geochemists to identify strongly-bound organic material in petroleum source rocks.

7.2 Methods

The exposure of volcanic ash from the Soufrière Hills volcano, Montserrat (SHV; ***Chapter 3***, *Section 3.2.1*) to a synthetic gas mixture that simulates exhaust emissions from a diesel vehicle was performed with the Emission Control Research group at the Johnson Matthey Technology Centre (JMTC), Reading, UK, and subsequently analysed in the laboratory, in the Chemistry Department, Durham University, as described in the following section.

7.2.1 Ash exposures to synthetic diesel exhaust gas emission

Three separate tests were performed on two occasions, to promote uptake of gases and compounds from a synthetic diesel mixture, specifically sulphur dioxide (SO_2), nitrogen dioxide (NO_2) and hydrocarbons (decane ($C_{10}H_{22}$) and toluene (C_7H_8)) (*Fig. 7-2*).

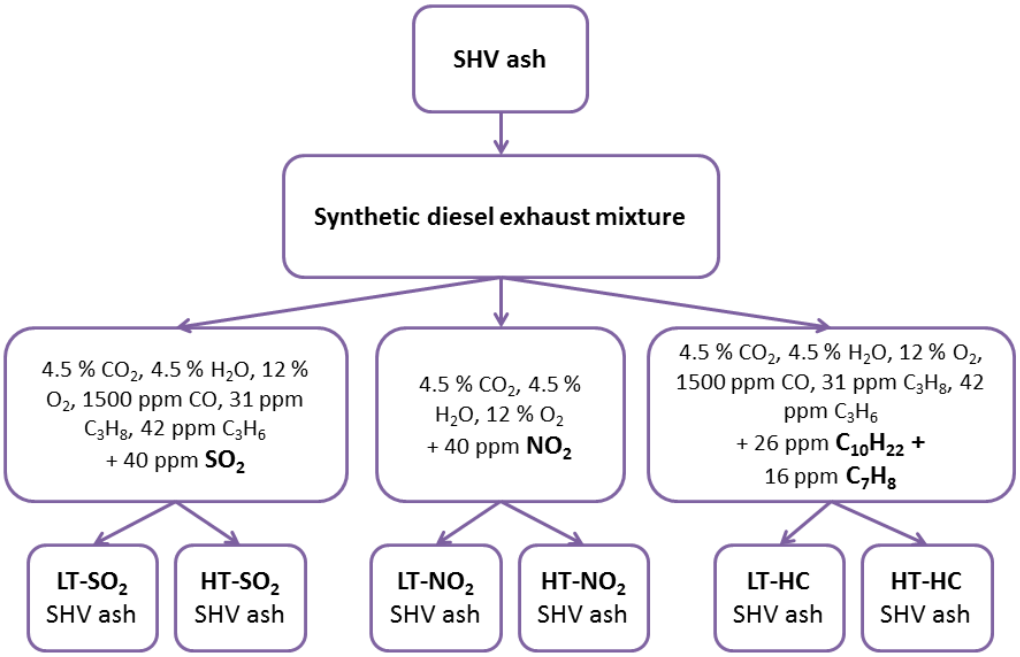

Figure 7-2: Schematic to demonstrate how Soufrière Hills (SHV) ash sub-samples were exposed to synthetic diesel exhaust of varying composition, at low temperature (LT) and high temperature range (HT).

The experiments[1] were performed using 100 mg of the ash per test, which was pelletised, crushed and sieved beforehand to give a fraction between 250 and 355 µm, as per the standard procedure of the laboratory (*e.g.*, Foo et al., 2015). The sample was loaded into a fixed-bed continuous flow reactor and exposed to a synthetic gas mixture, which contained 4.5 % carbon dioxide (CO_2), 4.5 % water (H_2O), 12 % oxygen (O_2), 1500 ppm carbon monoxide (CO), 31 ppm propane (C_3H_8), 42 ppm propene (C_3H_6) and the constituent under study (SO_2, NO_2 or $C_{10}H_{22}$+C_7H_8), at a total flow rate of 2 L/min. For the NO_2 test, the CO, C_3H_6 and C_3H_8 were removed to prevent further reactions occurring in the gas mixture.

The test rig was designed to replicate temperatures representative of those found along a vehicle exhaust pipe, from the manifold to tailpipe, in order to allow systematic testing of new catalytic converter materials. As such, experiments were conducted at two temperatures. During the 'high temperature' experiments, the sample was heated from 80 °C to up to 500 °C at a rate of 10 °C/min whereas, for

[1] *No further details about the experimental setup were provided to the author by JMTC.*

the 'low temperature' experiments, the temperature was held constant at around 80 °C, emulating exhaust gases at point of emission from a typical commercial vehicle exhaust. Lower temperatures were precluded owing to problems associated with condensation of water causing problems with the gas measurements, so these pilot experiments did not allow for testing of interactions at ambient temperatures, as may be expected within short distances of the vehicle tail pipe. The gas concentrations during the adsorption and desorption experiments were measured by *in situ* Fourier-transform infrared (FTIR) spectroscopy.

7.2.2 Pyrolysis gas chromatography-mass spectrometry

The original, untreated SHV ash, and the ash treated with simulated vehicle exhaust, which included $C_{10}H_{22}$+C_7H_8 in the mixture, at 80 °C (LT-HC, *Fig. 7-2*) were analysed using a Py-GCMS, in order to study any surface-adsorbed species, thereby confirming if gas uptake noted by FTIR occurred and was stable. Due to time constraints and limited availability of the equipment at the time, other samples could not be examined and this sample was prioritised based on the noted results.

For the analysis, the samples were weighed (~20 mg) into quartz sample tubes and packed using quartz wool. They were pyrolysed for 2 min at 300 °C using a Chemical Data System 5000 pyroprobe coupled with a 7820A gas chromatograph (*Agilent Technologies*, USA) interfaced with a 5977E mass selective detector (*Agilent Technologies*, USA). The oven temperature programme was 30 °C to 300°C at 50 °C/min. Compounds were identified using the *MassHunter Qualitative Analysis* software (version B.06.00, *Agilent Technologies*, USA) and the inbuilt NIST 11 mass spectral database.

7.3 Results

The profiles of gas adsorption at 'high temperatures' for SHV ash are plotted in *Fig. 7-3*, as measured by the FTIR during the exhaust exposure experiments. The only gas to show a clear uptake (signified by a lowering in signal intensity) was SO_2 between 300 - 450 °C (*Fig. 7-3A*) although some adsorption of NO_2 was indicated

at temperatures between 200 - 400 °C (*Fig. 7-3B*). In both cases, this was followed by another drop in signal intensity, at higher temperature, indicating that additional absorption may have occurred. No uptake of hydrocarbons was observed – the drop in the signal intensity once temperature of 300 °C was reached possibly indicates that the combustion of hydrocarbons occurred in the mixture (*Fig. 7-3C*).

For the 'low temperature' experiments, the concentration of SO_2, NO_2 and decane measured during individual exposures by the FTIR decreased at about the same time - just after one minute of exposure to the gas mixture at 80 °C - but then rapidly (within seconds) recovered to the initial signal intensity level (*Fig. 7-4*), suggesting gas storage saturation was reached on the ash. No adsorption of toluene was measured.

Py-GCMS analysis of the ash treated with simulated exhaust at 80 °C did not detect any of the adsorbed species of interest (*i.e.*, decane as noted by FTIR) coming off the particle surface, which was confirmed by comparison with the analysed untreated ash (***Appendix 1***).

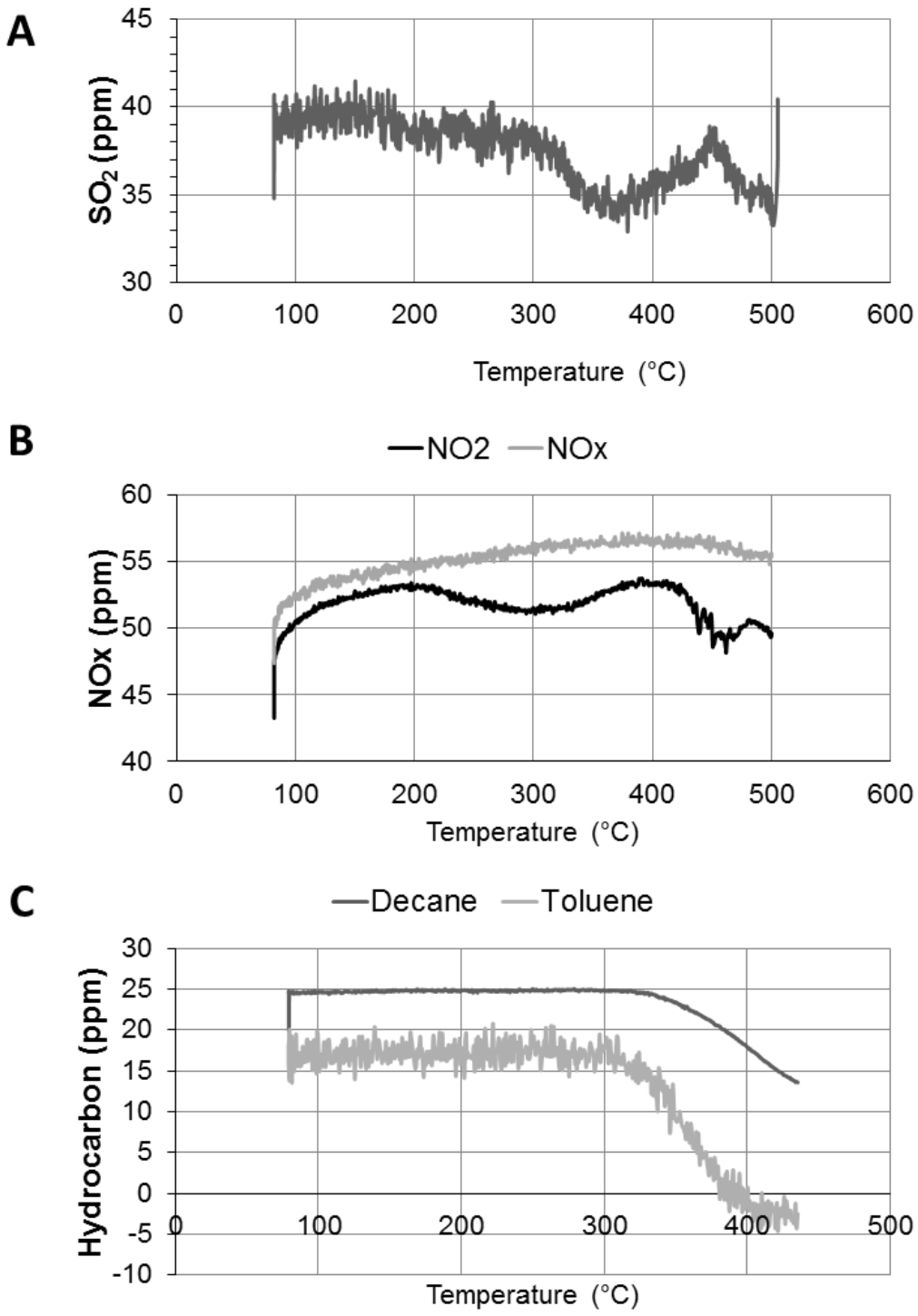

Figure 7-3: Gas uptake on volcanic ash over a high-temperature range. **A)** Sulphur dioxide (SO_2), **B)** nitrogen dioxide (NO_2) and oxides (NOx = NO+ NO_2), and **C)** hydrocarbons (decane and toluene) concentrations measured by FTIR. Baseline values were 40 ppm for SO_2 and NO_2, 26 ppm and 16 ppm for decane and toluene, respectively.

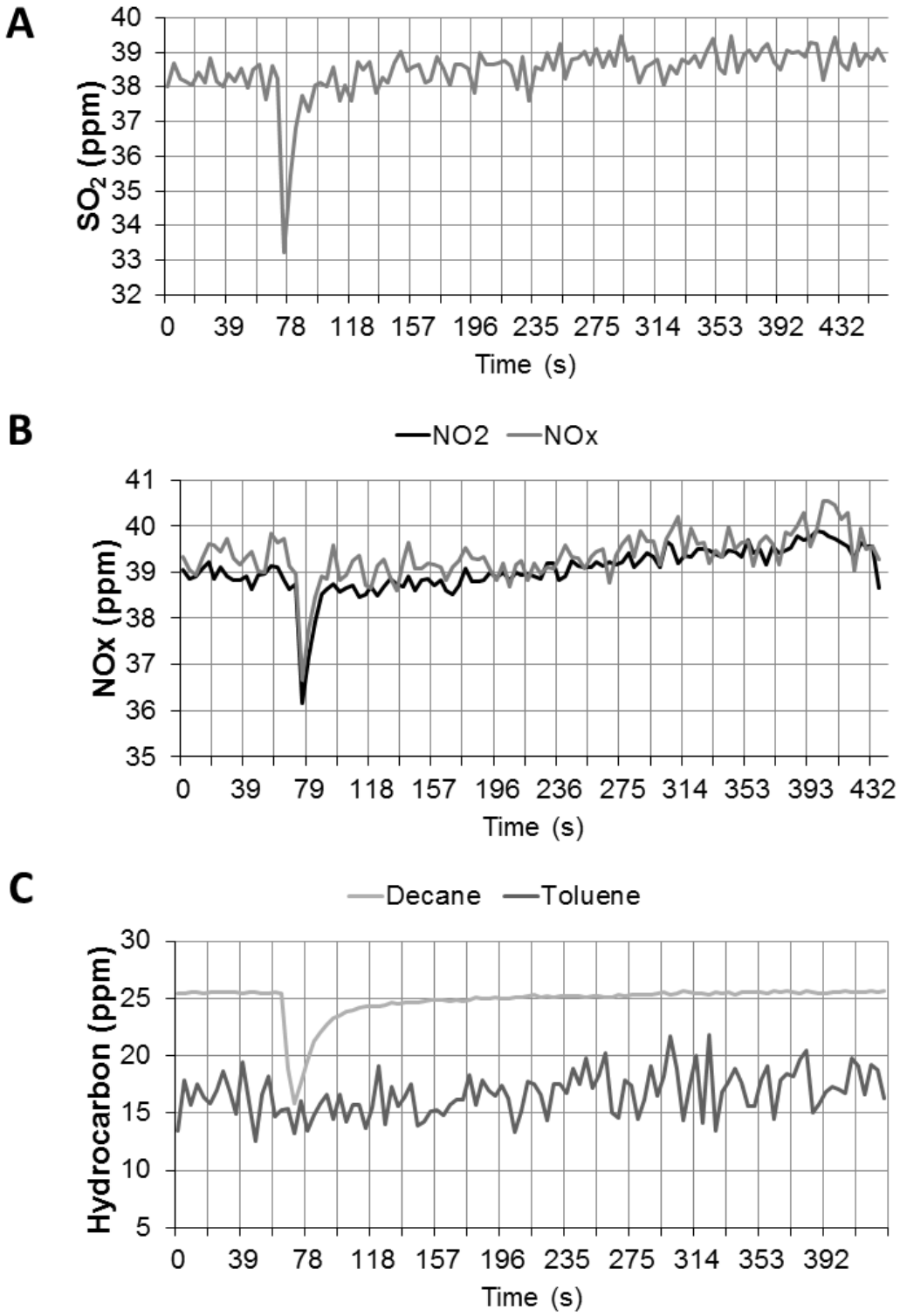

Figure 7-4: Gas uptake on volcanic ash as a function of time during exposure to synthetic diesel exhaust at 80°C. **A)** Sulphur dioxide (SO_2), **B)** nitrogen dioxide (NO_2) and oxides (NOx), and **C)** hydrocarbon (decane and toluene) concentrations measured by FTIR. Baseline values were 40 ppm for SO_2 and NO_2, 26 ppm and 16 ppm for decane and toluene, respectively.

7.4 Discussion

The nature and abundance of chemical functional groups on volcanic ash surfaces have been recently characterised, demonstrating that ash surfaces are multifunctional, combining acidic, basic, reduced and oxidised sites in various proportions, depending on ash composition (Maters et al., 2016). These sites are important in controlling ash reactivity, as the heterogeneous reactions and uptake of atmospheric/ambient gases depend on the amount and nature of the available surface sites on ash.

The following sections briefly discuss the potential ability of volcanic ash to uptake pollutant gases (from synthetic diesel exhaust) based on the results of the initial experiments conducted here. Since no surface reactivity analysis to assess available surface sites on the SHV ash nor an exhaustive post-exposure characterisation were performed due to time and technical constraints, some assumptions are made based on the knowledge available in the existing literature.

7.4.1 Sulphur dioxide uptake

Since SO_2 is an acidic gas, its uptake is believed to occur on basic surface sites, where reaction is likely to occur with oxide anions or hydroxyl groups, leading to formation of species containing sulphite (SO_3^{2-}) or bisulphite (HSO_3^-) (Goodman et al., 2001, Usher et al., 2002, Zhang et al., 2006).

The uptake of SO_2 on SHV ash observed during the exposures in this study was not surprising. With regards to the 'high temperature' experiments, the potential of ash to scavenge SO_2 in a hot volcanic plume (300-700 °C) has been established and described before (Ayris et al., 2013). In addition, we have observed efficient SO_2 uptake by synthetic volcanic glass and natural pumice at 500 °C, which resulted in emplacement of surface $CaSO_4$ deposits, in the study described in ***Chapter 4***.

Our results indicated some uptake of SO_2 at a lower temperature of 80 °C as well (*Fig. 7-4A*). Maters et al. (2017) previously demonstrated that ash is reactive toward atmospheric SO_2 at ambient temperature. Hence, it is reasonable to assume

that, even in the presence of a complex gas mixture, ash has the capacity to react with and adsorb SO_2. This implies that the ash exposed here could have been coated with, *e.g.*, sulphate salts, similarly to particles investigated in ***Chapter 4***. There, no adverse biological effects upon the *in vitro* lung model could be observed following particle exposures, suggesting that such species do not contribute to ash toxicity.

7.4.2 Nitrogen dioxide uptake

Studies on mineral oxide particles have shown that the uptake of NO_2 occurs by forming nitrites (NO_2^-) and, subsequently, nitrates (NO_3^-) on the particle surface, accompanied by some secondary reactions resulting in production of gaseous nitric oxide (NO) and nitrous acid (HONO) (Goodman et al., 1999, Underwood et al., 1999, Underwood et al., 2001, Finlayson-Pitts et al., 2003). To the author's knowledge, only a single study has considered NO_2 uptake by volcanic ash (Romanias et al., 2017). The findings of this recent study suggested that ash may be an important source of atmospheric HONO.

In the present study, a tendency of SHV ash to react with NO_2 at temperatures between 200 and 400 °C, as well as at 80 °C was observed (*Fig. 7-3B* and *7-4B*). The capacity for NO_2 uptake appears to be lower than for SO_2, which is in agreement with some previous findings where uptake of both gases on mineral particles was studied (Mamane and Gottlieb, 1989).

Although it is assumed that both sulphate and nitrate containing particles may contribute to particle toxicity upon inhalation, there is insufficient evidence to include or exclude them as being a component of ambient air particulate which is responsible for adverse health impacts (Reiss et al., 2007, Pope and Dockery, 2006).

7.4.3 Hydrocarbon uptake

Interaction and adsorption mechanisms of organic compounds onto mineral dust surfaces are not well understood and information on the reactions involved is limited (Usher et al., 2003b, Romanías et al., 2016). Some studies have found that organics (*e.g.*, acetaldehyde, acetone, propionaldehyde) adsorb through hydrogen-

bonding interactions with surface hydroxyl groups on SiO_2 particle surfaces, in a nonreactive and reversible way (Li et al., 2001, Carlos-Cuellar et al., 2003). A similar mechanism of interaction was discussed in a recent study by Romanías et al. (2016). The study investigated heterogeneous interactions of VOCs (limonene and toluene) with atmospheric Saharan dust samples. Their findings indicated that the adsorption capacities of the dust towards toluene increased with lower SiO_2 content, whereas samples with high SiO_2 content had a significantly lower reversible fraction (desorbed toluene).

Several organic compounds, predominantly hydrocarbons, have been identified adsorbed on volcanic ash (Lamparski et al., 1990, Stracquadanio et al., 2003, Takizawa et al., 1994) (see ***Chapter 2***, *Section 2.7*). Here, in this present study, some uptake of decane after exposures at 80 °C was observed (*Fig. 7-4C*); however, this was not confirmed with Py-GCMS analysis.

Decane is a saturated linear hydrocarbon, representative of uncombusted/partially combusted diesel fuel and is nonpolar with a boiling point of 174.1 °C. Owing to its lack of polarity, or any functional groups, without activation decane cannot undergo chemical reactions other than cracking and, as such, is expected to be mainly physisorbed at organophilic sites on ash particles as the main mode of interaction. There is much debate about the relative hydrophobic/organophilic properties of silicate surfaces when dehydrated (Lowen and Broge, 1961), so adsorption at raised temperatures would seem more likely than at low temperatures when rehydration of the surfaces may render them organophobic.

Toluene is an aromatic hydrocarbon compound (boiling point of 110.6 °C) often added to diesel fuels as an octane booster. Owing to its negative health implications (Cohr and Stokholm, 1979, EPA, 2005), toluene may be of concern if it is stabilised and delivered to the body in high concentrations on ash particles. Owing to its aromatic chemistry, toluene may interact with the ash surface in different ways to the saturated decane molecules. The delocalised pi (Π) electrons on the aromatic ring of the toluene are able to interact with electron deficient

regions of mineral surfaces. For example, Π electron-cation interactions have been observed at mineral surface on clay minerals (Mortland, 1970, Keiluweit and Kleber, 2009). Toluene may also behave in a way representative of more complex aromatic molecules such as PAH. However, no evidence was found for toluene-ash interactions in this pilot investigation.

In summary, it is likely that decane was weakly bound (physisorbed) on the ash surface during the reactor trials, and that it could not be detected post-experiment because it has desorbed during rehydration of the ash at lower temperatures (*Fig. 7-1A*). However, this cannot be confirmed, as no studies on volcanic ash, to date, have examined or discussed the potential reactions with organic compounds or their products. Based on these initial experiments, it is also difficult to state whether the ash surface following exposure to diesel exhaust can be modified in a way that could potentially change its toxicity. Since crystalline silica is a significant component of volcanic ash, it would be relevant to also assess the interaction between crystalline silica and diesel exhaust. Moreover, since some previous studies which investigated co-exposures to crystalline silica and diesel particulate matter (DEP) *in vivo* found a potential increased risk for adverse health effects in concomitant exposure to silica and DEP (Farris, 2017).

Since it has been recognised that modifications to particle surface chemistry that stem from adsorption of species from the urban environment, such as VOCs, can affect particle-cellular interactions and consequently, clearance and translocation processes in the lung (Sun et al., 1984, Fubini, 1997, Knaapen et al., 2004, Saptarshi et al., 2013), it is clear this area requires further research in order to broaden our understanding of such interactions and their impact on ash toxicity.

7.4.4 Experimental approach

For this study, a number of methods which could potentially be applied to study ash-exhaust interactions were investigated. The test rig finally used in the experiments (*Section 7.2.1*) is, in fact, a commercial system designed to test catalytic converters (*i.e.*, emission control devices used to detoxify gaseous pollutants from vehicle exhaust). Although the system is sophisticated, including its

ability to emulate different exhaust gases and an *online* analysis, it had limitations for application in the present study. Primarily, access was restricted, since it is within a commercial facility and, hence, only SHV ash was analysed and uptake was only investigated on a single replicate for different exposure scenarios. Consequently, it was not possible to use a range of ash samples representative of different magmatic origins, which may react differently with gases (Maters et al., 2016), nor account for potential differences in gas uptake due to the heterogeneity of natural ash samples by using multiple replicates. It was also not possible to study the effect of sequences of different treatments, for example taking ash contacted with SO_2 and then subsequently exposing it to hydrocarbon gases.

Furthermore, the design of the rig to emulate the manifold to the exhaust tailpipe, and the coressponding temperature range, could potentially be of interest in studies with different aims (*e.g.*, if particles are likely to pass through the engine) but, here, the aim was to simulate the interactions at ambient temperature. Hence, after having used this system, a different test bed system at a BP (UK) facility was identified, where real engine exhaust, from different vehicle types, can be produced and potentially used to expose ash at more representative ambient conditions in a ducting system on the facility roof. However, this system is accompanied by only portable gas flow testers, which would make the accuracy of gas analysis more challenging. No further experiments were performed within the timeframe of the present doctoral study though links have been made to follow up in the future.

Future studies could consider *e.g.*, exposing ash to a real, complete vehicle exhaust using an (adapted) exposure system such as described in ***Chapter 6*** (*Sections 6.2.1* and *6.2.2*), accompanied by particle surface characterisation techniques (post-exposure). Alternatively, a Knudsen flow reactor could be used (*e.g.*, Caloz et al., 1997), which utilises synthetic pollutant gas and/or gas mixtures and is commonly applied in studies of heterogeneous chemistry of solid substrates, allowing characterisation of surface functional groups present on particles. An experimental setup combining diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, selected-ion flow-tube (SIFT) mass spectrometry and long

path FTIR spectroscopy, which allows monitoring of both the adsorbed and gas phases in real time (Romanías et al., 2016, Zeineddine et al., 2017) could be used as well.

7.5 Conclusion

This study was the first to investigate the uptake of gaseous species from a simulated diesel exhaust onto volcanic ash surfaces. It has been observed that volcanic ash can adsorb SO_2 and NO_2 from a complex mixture, whereas the capacity for hydrocarbon uptake was not clear under the present experimental conditions. There is some evidence that decane, a linear hydrocarbon, was adsorbed at higher temperatures, however it is not clear if this remains adsorbed or rapidly desorbs. Overall, these findings 'confirm' the previously demonstrated ability of ash to react with, and scavenge, volcanic and anthropogenic gases.

More detailed investigations of these interactions are necessary, especially to deduce the capacity of ash to scavenge urban pollutants at realistic, ambient temperature and conditions. In addition, appropriate surface techniques (such as XPS, infrared and Raman spectroscopy) could then be used to assess the characteristics and products of the interactions, thus giving an insight into the toxic potential of such particles.

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Part IV

Conclusion

Chapter 8

Implications and Conclusions

8.1 Introduction

The potential adverse health outcomes of exposure to inhalable volcanic ash have been a long-standing concern (Horwell and Baxter, 2006) and this is more pertinent now that inhalable ambient particulate matter has been classified as a lung carcinogen (Loomis et al., 2013). The uncertainties of 'if' and 'how' external factors, including the volcanic plume, itself, and anthropogenic pollutants, may alter ash toxicity and contribute to any adverse respiratory health effects, has been outlined in recent reports (Loughlin et al., 2012, Kar-Purkayastha et al., 2012). Therefore, this thesis set out to determine, for the first time, whether ash particle coatings, which originate from in-plume reactions with volcanic gases, can contribute to or alter ash toxicity (***Part II***) as well as if concomitant exposure to volcanic and anthropogenic pollutants poses a greater respiratory hazard than the individual respiratory toxicities of either anthropogenic pollution or volcanic ash alone (***Part III***). This was done by testing the hypotheses outlined in ***Chapter 1***, mainly through an *in vitro* toxicological approach.

It was found that the adsorbed species constituting coatings on simulated ash surfaces (*e.g.*, sulphate salts) had no adverse short-term biological impact to a multicellular lung model (***Chapter 4***), suggesting that the studied in-plume processes likely do not affect the toxicity of volcanic ash. Combined exposure to respirable-sized volcanic and diesel exhaust particles was shown to induce a heightened (pro-)inflammatory response *in vitro*, implying a potentially-greater hazard of simultaneously inhaling both particle types (***Chapter 5***). A lack of such effect was seen, however, during exposure to complete gasoline exhaust in the presence (or absence) of volcanic ash, regardless of the ash bulk magmatic composition (***Chapter 6***). Finally, pilot work on the potential of ash to uptake pollutant gases originating from a vehicle exhaust was conducted (***Chapter 7***), but further work is necessary to reveal whether such particles, acting as carriers of potentially harmful adsorbed species, could induce a different toxicological response *in vitro* (see *Section 8.4*).

8.2 Implications for understanding the toxicity of volcanic ash

In ***Chapter 4*** of this study, it was found that, upon contact with alveolar tissue structures, including surfactant and cells, water-soluble species from ash surface coatings are likely to rapidly dissolve. Therefore, the presence of salt coatings was shown to have no adverse biological impact to cells (***Chapter 4***). Thus, contrary to *Hypothesis 1*, it appears that surficial $CaSO_4$ salts and, hence, this specific type of in-plume processing, do not alter volcanic ash toxicity. These findings have potential implications for the reported short-term human health effects following eruptions. It is deemed unlikely that particles with coatings are specifically responsible for acute respiratory effects, since the observed *in vitro* effects are largely comparable with those seen following *in vitro* exposures to pristine ash.

From these findings, alone, it is difficult to determine whether such particles are the reason behind the reported acute irritancy to the airways (Horwell and Baxter, 2006, Witham et al., 2005). It could be possible, however, that volcanic particles contribute to the effects of inhaling gaseous volcanic sulphur dioxide, a known respiratory irritant (Hansell and Oppenheimer, 2004), and acid aerosols during an eruption, but the biological impacts of such co-exposures warrant further studies.

From the direct comparison of their individual respiratory toxicities *in vitro*, this study has shown that ash (from Soufrière Hills volcano, Montserrat) is less toxic than diesel exhaust particles, which were used as representative of a common, combustion-derived, constituent of ambient urban air (***Chapter 5***). This indicates the likelihood that volcanic ash is of lower toxicity than particulate matter in the urban air, as postulated by a limited number of the existing studies (see Kar-Purkayastha et al., 2012), and, thus, offers a valuable addition to the knowledge on which public health decision-making can be based.

When co-exposed, however, volcanic ash and diesel exhaust particles were found to induce a heightened (pro-)inflammatory response *in vitro*, greater than the response noted for each particle type separately (***Chapter 5***). Thus, the findings of this study support *Hypothesis 2* and it can be reasonably stated that combined

exposure to volcanic ash and diesel exhaust particulate poses a greater respiratory hazard than exposure to either, independently. The observed responses can be largely attributed to the effect of the greater combined dose of particles delivered to the cell surface, which means that the potential health impact is likely to be similar to that expected to result from the increased concentration of particulate matter in the air. The importance of these findings lies in the potential effects on respiratory health that such combined exposure may elucidate, if the exposure persists, since it is known that increased release of (pro-)inflammatory mediators may augment, as well as prolong, inflammatory reactions (Schwarze et al., 2013). Airway inflammation promotes the development of lung diseases, but it may even increase the susceptibility to acute cardiovascular disease (Donaldson et al., 2001).

Contrarily, the combined exposure to complete gasoline exhaust and volcanic ash caused no adverse effects to cells *in vitro* and did not result in either an additive or synergistic response (***Chapter 6***). Furthermore, in discordance with *Hypothesis 3*, the outcome of these exposures was not influenced by differences in volcanic ash types, such as composition and mineralogy. This, however, does not dismiss the possibility that a different exhaust type and a different sample from an individual eruption, or a different ash type, might incite a different response. These findings, therefore, emphasise the necessity to take into consideration the complexity and variability of components of ambient urban air, as well as the heterogeneity of natural ash samples, when assessing the potential respiratory hazard of combined exposures.

Although the multicellular system used throughout this thesis has its advantages (*e.g.*, providing a realistic inter-cellular signalling amongst different cell types which will influence the endpoints measured), it was not shown to be better (or worse) than monocultures, considering that the outcomes of this study are largely comparable to findings of previous studies on volcanic ash toxicity which have used monoculture systems (Damby et al., 2016, 2013, Horwell et al., 2013, Wilson et al., 2000, Cullen and Searl, 1998). It is, however, difficult to state, especially without comparative *in vitro* and *in vivo* studies, whether one is more

optimal than the other for use in the assessment of the potential hazard of volcanic ash.

The experiments in ***Chapter 7*** indicated that volcanic ash is capable of scavenging urban pollution gases, however, whether this has resulted in the formation of 'unusual' surface compounds, was not fully addressed in the present study (*Hypothesis 4*). This should be the focus of future work, which could then assess whether such interactions may affect the toxic potential of volcanic ash.

8.3 Implications for hazard management

The findings of this thesis provide a valuable first assessment of potential impacts of external factors on the toxicity of volcanic ash and, as such, provide a useful source of preliminary information to be used by governmental agencies and hazard management organizations, worldwide, in the event of a volcanic eruption. This is especially the case because, currently, there is a lack of epidemiological studies that consider health effects of ashfall in urban areas affected by the existing high levels of air pollution. It has to be noted that the studies in this thesis considered short-term exposure effects, while long-term effects require further work (*Section 8.4*).

A recent World Health Organization review on the health effects of exposure to particulate air pollution noted that crustal particles (which they classified as being coarse grained; $PM_{2.5-10}$) may be as toxic as (urban) $PM_{2.5}$ on a mass basis (WHO, 2013). Considering this finding, volcanic ash could beconsidered to have an equivalent toxicity to $PM_{2.5}$ in ambient urban air, although a greater evidence base is required before this could be confirmed and, given the findings of toxicological research, to date, including in this thesis, it seems unlikely. In the absence of this evidence, however, civil protection agencies may wish to take a precautionary approach with public health guidance, especially if combined exposure to ash and urban PM potentially poses an increased hazard toward respiratory health. Agencies could advise that citizens should minimise or avoid exposure especially during periods of intense exposure to both urban pollution and volcanic ash.

8.4 Future work

This study was the first to address the effects of in-plume processing on volcanic ash toxicity. Although the analogue experiments indicated that the presence of salts does not augment ash toxicity, only the effects of the predominant adsorbed phase (sulphate salts) have been examined. Adsorption of volatiles in the eruption plume, and in the atmosphere, is controlled by various factors, such as magma type, eruption style, particle size, environmental conditions, etc. (Witham et al., 2005), thus, influencing the nature and abundance of surface coatings. The impact of, *e.g.*, chloride and fluoride salt coatings stills needs to be investigated.

In the present study, chloride coated particles formed aggregates during the experiments, thereby affecting the recovery of the respirable fraction making toxicological analysis impossible (of that fraction). To overcome these issues, future studies could perhaps attempt to use the 'bulk' sample to get an insight into the potential biological impact, as has been done in previous studies (*e.g.*, Damby et al., 2013, Horwell et al., 2013), since it was shown that bulk and respirable samples give similar results, at least with the haemolysis assay (Damby, 2012). Another possibility could be to use particle leachate solutions in *in vitro* assays (*i.e.*, using cultures not at the ALI), which also allows the contribution of soluble components to be constrained (*e.g.*, Nattrass et al., 2017).

Further examination of the mechanisms driving the heightened (pro-)inflammatory response following combined exposure to volcanic ash and diesel exhaust particles should be carried out, as is not yet clear whether this effect is driven by the individual particle-cell interactions related to the higher dose, secondary toxicology mechanisms incited via the particles' physicochemical characteristics, or through particle-particle interactions which then interact with cells.

Since only the effects of andesitic ash and diesel exhaust particles were compared here, investigation and comparison of the relative toxicity of different ash types with diesel exhaust particles, as well as with other particles from the urban air (*e.g.*, road dust), would be useful to assess if the biological response may

vary. Similarly, a different fuel/engine type could be used (*e.g.*, diesel) to clarify the hazard posed by combined exposures to complete exhaust. Additional biological markers relevant for inducing respiratory diseases could also be considered in further experiments *in vitro*, such as genotoxicity, which can be induced by particles even in the absence of inflammation (Schins, 2002), to investigate whether co-exposures with ash may potentially cause cellular DNA damage or augment the known genotoxic potential of DEP (IARC, 1989).

More detailed investigations of the interactions of ash with ambient pollution are necessary, especially to deduce the capacity of ash to scavenge urban pollutants at realistic, ambient temperatures and conditions as this was not possible during this study. As discussed in ***Chapter 7***, future studies could consider exposing ash to a real, complete vehicle exhaust in a customised exposure system or utilise techniques commonly applied in studies of the heterogeneous chemistry of solid substrates (*e.g.*, Knudsen reactor). In addition, appropriate surface techniques (such as XPS, infrared and Raman spectroscopy) could then be used to assess the characteristics and products of the interactions, thus giving an insight into the formation of such particles and, hence, their potential toxicity.

Potential chronic effects of the exposure scenarios described in this thesis, which may occur over a prolonged period, have not been accounted for and need to be investigated. In ***Chapter 5***, it was shown that ash has the potential to incite an inflammatory response, as evidenced in the cell exposure experiments by an increase in secretion of (pro-)inflammatory cytokines. The potential to incite a chronic inflammatory effect could be assessed using an *in vivo* approach, which would also allow estimation of particle clearance from the lung and dissolution of particles in actual lung fluid.

The effects of exposures to urban pollutants and ash on people suffering from pre-existing respiratory diseases, who are likely to be more susceptible to elevated ambient particle concentrations, should also be determined. For this purpose, *in vitro* studies employing cells from both healthy and asthmatic donors could be utilised (*e.g.*, Chortarea et al., 2017).

8.5 Concluding remarks

The doctoral research presented in this thesis investigated, for the first time, whether volcanic ash toxicity is impacted by combined exposures with urban pollution or through the interactions encountered with gases within the volcanic plume. The key finding of this thesis is that combined exposure to volcanic ash and diesel exhaust particulate causes a heightened (pro-)inflammatory response, *in vitro*, and thus, appears to pose a greater respiratory hazard than the individual respiratory toxicities of either diesel exhaust particulate or volcanic ash. However, no significant toxicological effects of in-plume processing or co-exposures with complete (gasoline) exhaust were found. The fact that sulphate salts dissolve rapidly, likely prior to cellular uptake, is a critical finding which helps explain why the salt-laden samples had no toxicological impact.

Further investigations are now required to derive a more comprehensive understanding of the interactions of volcanic ash and urban pollutants in the ambient air and potential impacts of co-exposures, including the interactions of ash and complete diesel exhaust, the potential chronic effects over a prolonged period of exposure, as well as effects such exposures could elucidate in individuals with pre-existing respiratory diseases. Still, these initial findings provide the first evidence which can be used towards the assessment of respiratory health hazard following the onset of new volcanic activity where exposed communities live in heavily polluted urban areas.

8.6 References

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Appendix 1

Supporting data

Water and acid leach of SHV and ChV ash samples

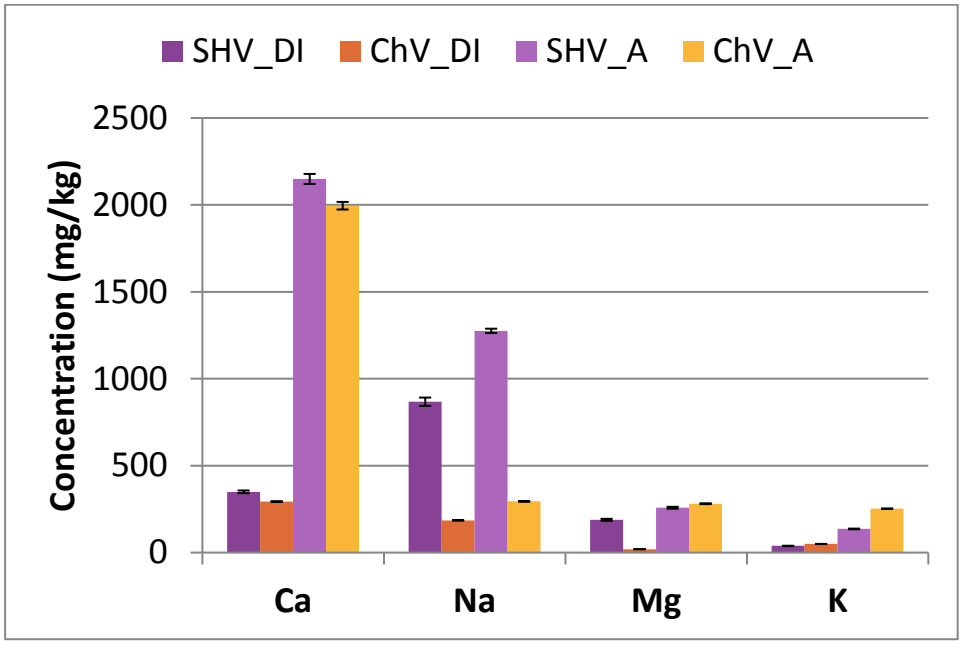


Fig. A-1: Concentrations of major elements in deionized water (DI) and acid (A) leach of SHV and ChV ash. Samples were leached at a ratio 1:20. The error bars are the standard error of the mean (n=4 and n=3 for SHV and ChV, respectively).

Table A-1: Concentrations of minor elements in deionized water (DI) and acid (A) leach of SHV and ChV ash. Samples were leached at a ratio 1:20. SD = standard deviation, SEM = standard error of the mean (n=4 and n=3 for SHV and ChV, respectively).

	Al	Mn	Fe	Co	Ni	Cu	Zn	As	Pb
SHV_DI	**4.66**	**28.78**	**0.00**	**0.19**	**0.00**	**1.48**	**1.65**	**0.00**	**0.00**
SD	0.25	1.90	0.00	0.01	0.00	0.08	0.12	0.00	0.00
SEM	0.12	0.95	0.00	0.01	0.00	0.04	0.06	0.00	0.00
SHV_A	**1956**	**39.22**	**604**	**0.31**	**0.01**	**4.73**	**2.14**	**0.11**	**0.22**
SD	51.20	2.13	14.69	0.02	0.01	0.27	0.27	0.00	0.00
SEM	25.60	1.06	7.34	0.01	0.00	0.13	0.13	0.00	0.00
ChV_DI	**1.35**	**3.94**	**0.87**	**0.00**	**0.01**	**0.01**	**0.03**	**0.53**	**0.00**
SD	0.35	0.05	0.11	0.00	0.00	0.00	0.05	0.01	0.00
SEM	0.20	0.03	0.06	0.00	0.00	0.00	0.03	0.00	0.00
ChV_A	**528**	**63.01**	**679**	**0.17**	**0.20**	**0.60**	**4.19**	**5.55**	**1.04**
SD	2.22	2.05	18.91	0.00	0.00	0.02	0.10	0.08	0.06
SEM	1.28	1.18	10.92	0.00	0.00	0.01	0.06	0.05	0.04

Water leach of SHV and ChV ash samples in two complementary tests at different S/L ratios

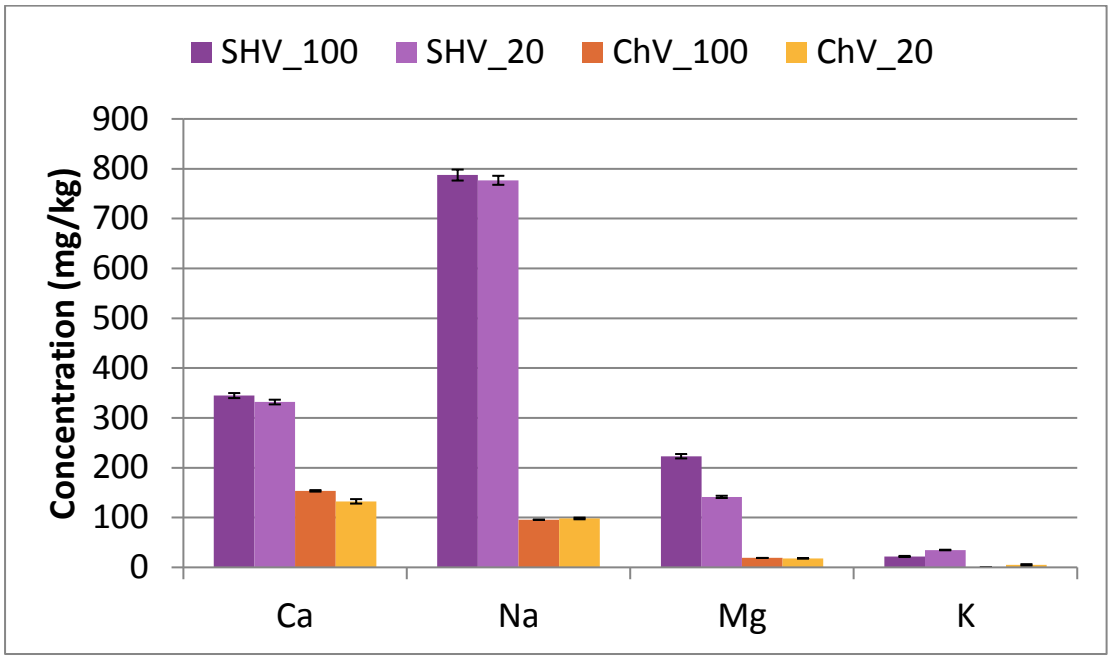


Fig. A-2: Concentrations of major elements in deionized water leach of SHV and ChV ash. Samples were leached at ratios 1:100 and 1:20. The error bars are the standard error of the mean (n=3).

Table A-2: Concentrations of minor elements in deionized water leach of SHV and ChV ash. Samples were leached at ratios 1:100 and 1:20. SD = standard deviation, SEM = standard error of the mean (n=3).

	Al	Mn	Co	Cu	Zn	As	Sr	Ba	Li	B
SHV_100	**1.91**	**32.21**	**0.15**	**1.48**	**1.87**	**0.01**	**0.71**	**0.05**	**0.14**	**0.10**
SD	0.27	0.96	0.00	0.03	0.68	0.00	0.02	0.01	0.01	0.02
SEM	0.15	0.56	0.00	0.02	0.39	0.00	0.01	0.00	0.00	0.01
ChV_100	**2.91**	**2.92**	**0.00**	**0.00**	**0.00**	**1.05**	**0.27**	**0.08**	**0.11**	**0.87**
SD	0.16	0.06	0.00	0.00	0.00	0.06	0.01	0.00	0.00	0.03
SEM	0.09	0.04	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.02
SHV_20	**2.16**	**24.03**	**0.15**	**1.69**	**2.56**	**0.00**	**0.68**	**0.04**	**0.12**	**0.10**
SD	0.16	0.59	0.00	0.03	0.86	0.00	0.02	0.00	0.00	0.01
SEM	0.09	0.34	0.00	0.02	0.50	0.00	0.01	0.00	0.00	0.00
ChV_20	**6.35**	**2.33**	**0.00**	**0.01**	**0.21**	**0.67**	**0.26**	**0.18**	**0.09**	**0.81**
SD	4.87	0.42	0.00	0.01	0.20	0.10	0.03	0.12	0.01	0.03
SEM	2.81	0.24	0.00	0.00	0.11	0.06	0.02	0.07	0.00	0.02

Table A-3: Concentrations of anions in deionized water leach of SHV and ChV ash. Samples were leached at ratios 1:100 and 1:20. SD = standard deviation, SEM = standard error of the mean (n=3).

	F^-	Cl^-	SO_4^{2-}	NO_3^-
SHV_100	-	1825	1388	23980
SD	-	220	48	39321
SEM	-	127.22	27.92	22702.14
SHV_20	23.28	1869	839	190
SD	2.77	68	35	2
SEM	1.60	39.49	20.45	1.11
ChV_100	-	765	894	45434
SD	-	60	51	38792
SEM	-	34.67	29.27	22396.84
ChV_20	25.44	432	235	195
SD	1.05	62	2	4
SEM	0.61	35.95	0.90	2.03

Simulated lung fluid leach – cell culture medium proxy

Table A-3: RPMI 1640 cell culture medium composition.

Components	Concentration (mg/L)
Amino acids	
Glycine	10.0
L-Arginine	200.0
L-Asparagine	50.0
L-Aspartic acid	20.0
L-Cystine 2HCl	65.0
L-Glutamic Acid	20.0
L-Glutamine	300.0
L-Histidine	15.0
L-Hydroxyproline	20.0
L-Isoleucine	50.0
L-Leucine	50.0
L-Lysine hydrochloride	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline	20.0
L-Serine	30.0
L-Threonine	20.0
L-Tryptophan	5.0
L-Tyrosine disodium salt dihydrate	29.0
L-Valine	20.0
Vitamins	
Biotin	0.2
Choline chloride	3.0
D-Calcium pantothenate	0.25
Folic Acid	1.0
Niacinamide	1.0
Para-Aminobenzoic Acid	1.0
Pyridoxine hydrochloride	1.0
Riboflavin	0.2
Thiamine hydrochloride	1.0
Vitamin B12	0.005
i-Inositol	35.0
Inorganic salts	
Calcium nitrate ($Ca(NO_3)_2$ $4H_2O$)	100.0
Magnesium Sulfate ($MgSO_4$) (anhyd.)	48.84
Potassium Chloride (KCl)	400.0
Sodium Bicarbonate ($NaHCO_3$)	2000.0
Sodium Chloride (NaCl)	6000.0
Sodium Phosphate dibasic (Na_2HPO_4) anhydrous	800.0
Other components	
D-Glucose (Dextrose)	2000.0
Glutathione (reduced)	1.0
Phenol Red	5.0

Particle exposures to HCl in the Advanced Gas-Ash reactor (AGAR)

Volcanic glass sample (*Section 4.2.1*) was loaded into the quartz sample bulb and placed within the horizontal tube furnace (*Section 4.2.2*) and heated to a temperature of 500°C. The experiments were carried out under a 25 SCCM gas stream of HCl and 75 SCCM of Ar carrier gas, exposing ash for a total of 120 min.

The material recovery during separation of near-respirable fraction (***Chapter 3***, *Section 3.3.1*) for toxicological analysis was negligible. Visualisation of the sample by SEM showed aggregation of particles (*Fig. A-1*), which most likely occurred during the experiment via cementation of salts between particles.

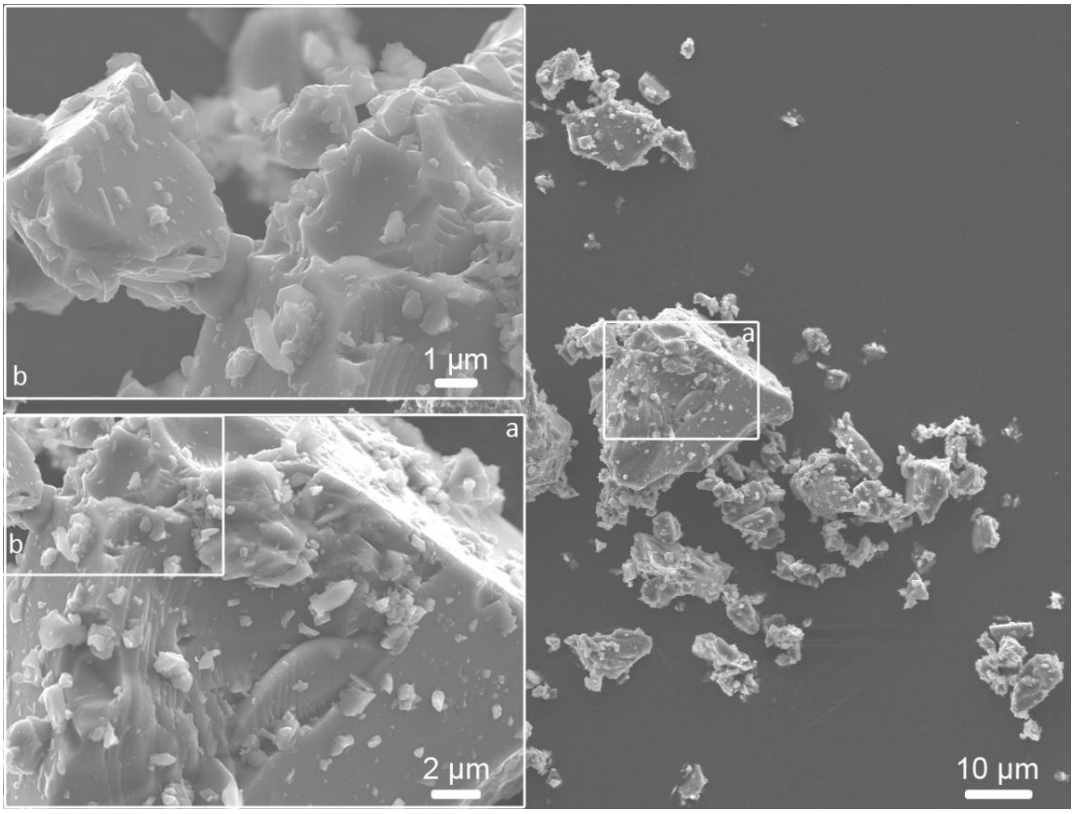


Figure A-3: Representative scanning electron micrographs of synthetic volcanic glass particles exposed to HCl for 2 h in the Advanced Gas-Ash Reactor (AGAR). Images were collected at 10.0 kV and 8.1 mm working distance. Scale bars are noted on the images.

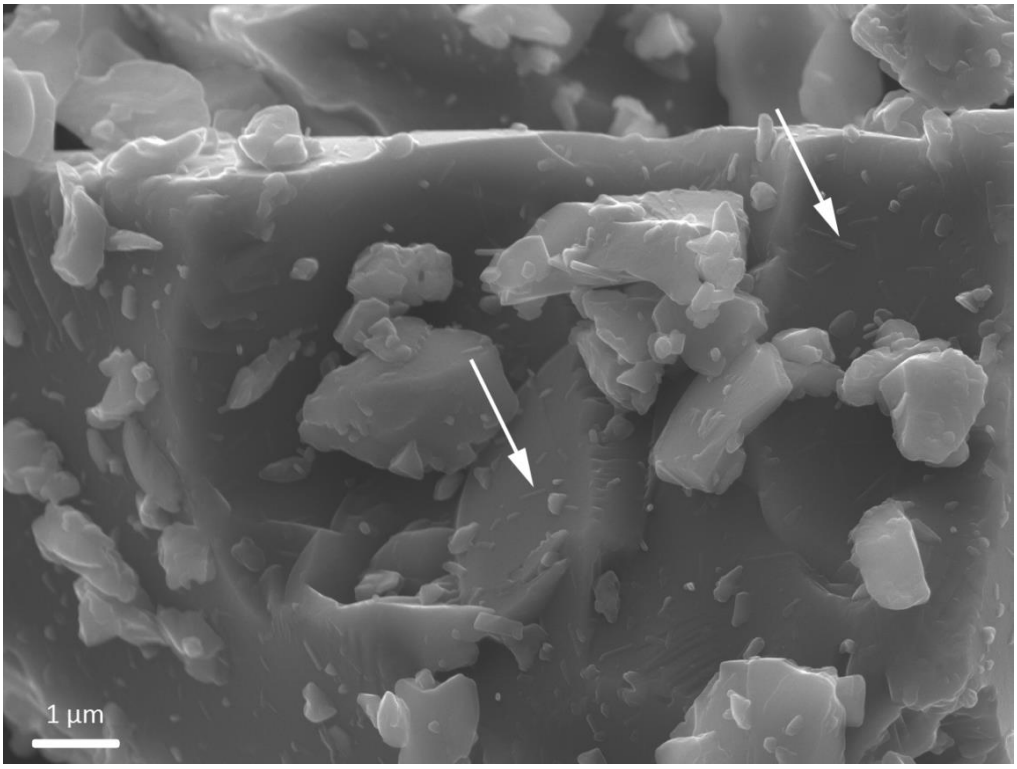


Figure A-4: Representative scanning electron micrograph of synthetic volcanic glass particles exposed to HCl for 2 h in the Advanced Gas-Ash Reactor (AGAR). White arrows indicate needle-like crystal surface features. Image was collected at 10.0 kV and 8.4 mm working distance. Scale bar is noted on the image.

Particle exposures to the mixed SO_2-HCl atmosphere in the AGAR

During a research stay at the Department of Earth and Environmental Sciences, Ludwig Maximilians University Munich (LMU), Germany, under the guidance of Dr Paul Ayris, some test experiments were performed where pumice sample (*Section 4.2.1*) was exposed to the mixed SO_2-HCl atmosphere under varying experimental conditions, in order to emplace salts (sulphates and halides) on the particle surface.

Around 2 g of sample per run was loaded into the quartz sample bulb and placed within the horizontal tube furnace and heated to a temperature of 500°C. All experiments were carried out under a 25 SCCM gas stream of SO_2 in Ar (525 SCCM of Ar carrier gas), and then 25 SCCM of HCl in Ar, exposing pumice ash sample to gases for varying period of time (*Table A-1*).

Subsequently, samples were leached in water as described in *Section 4.2.5*, in order to identify the species adsorbed onto the surfaces of particles during the experiments. Briefly, 0.2 g of the post-AGAR (bulk) samples were leached with deionised water at a ratio of 1:100 (g dry weight ash to mL water), each with a contact time of 1 hour at room temperature in static conditions, in one replicate. The concentrations of readily water-soluble surface cations (Ca, Na) and anions (SO_4, Cl) were then measured in the extracts using ICP-MS and IC, respectively (*Table A-1*).

Table A-4: Experimental conditions of pumice exposures to the mixed SO_2-HCl atmosphere in the AGAR and concentrations of water-soluble cations and anions (mg/kg), determined post-exposures.

Exposure time (min)		Sample code	Concentration (mg/kg)			
SO_2	HCl		Ca	Na	SO_4	Cl
60	15	**IT2**	1633	187	4941	152
120	15	**IT3**	1752	177	5151	118
30	15	**IT4**	1201	139	3411	202
60	30	**IT5**	971	120	2661	345
120	30	**IT6**	1963	170	5511	129
30	30	**IT7**	1489	150	4131	218
60	60	**IT8**	1618	127	4341	149
30	60	**IT9**	1643	146	4491	184
120	60	**IT10**	2235	171	6171	114

Biosolubility of particle salt coating – PHREEQC modelling

Initial solution for water dissolution test: pure water at 25 °C, equilibrated with CO_2, pH 5.98

Initial solution for SLF dissolution test: reagents dissolved in water at 37 °C, pH fixed at 7.4

Saturation indices for phases in SLF

```
  Phase               SI** log IAP   log K(310 K,   1 atm)

  Anhydrite         -6.97    -11.39   -4.42  CaSO4
  Aragonite         -1.52     -9.94   -8.42  CaCO3
  Calcite           -1.38     -9.94   -8.56  CaCO3
  CH4(g)             0.77     -2.12   -2.89  CH4
  CO2(g)            -3.50     -5.10   -1.60  CO2  Pressure   0.0 atm,
phi 1.000
  Dolomite          -2.90    -20.26  -17.36  CaMg(CO3)2
  Fix_H+            -7.40     -7.40    0.00  H+
  Gypsum            -6.80    -11.39   -4.59  CaSO4:2H2O
  H2(g)             -5.03     -8.16   -3.12  H2
  H2O(g)            -1.21     -0.00    1.21  H2O
  H2S(g)            -3.00    -10.97   -7.97  H2S
  Halite            -3.50     -1.92    1.58  NaCl
  Hydroxyapatite     7.27      2.83   -4.45  Ca5(PO4)3OH
  O2(g)            -69.38    -72.35   -2.97  O2
  Sulfur            -3.82      0.79    4.61  S
  Sylvite           -4.57     -3.62    0.96  KCl

**For a gas, SI = log10(fugacity). Fugacity = pressure * phi / 1
atm.
  For ideal gases, phi = 1.
```

Input parameters for dissolution reaction: measured concentrations of Ca, Na and Mg from water-leach data for 10, 30 and 60 min end-points for $CaSO_4$_G sample (*Table A-2*).

Table A-5: Input parameters for water and SLF dissolution simulations in PHREEQC.

CaSO4_G	Water leach data					PHREEQC calculation				
	Concentration (mmol/L)					water			SLF	
Time (min)	SO_4^{2-}	Ca^{2+}	Na^+	Mg^{2+}	Charge balance	$SI_{anhydrite}$	SO_4	pH final	$SI_{anhydrite}$	pH final
10	0.5303	0.4375	0.0912	0.0798	0.065	-2.53	0.5737	6	-5.76	7.57
30	0.5743	0.4819	0.0966	0.0847	0.081	-2.46	0.6228	6	-5.7	7.58
60	0.5913	0.5010	0.1053	0.0894	0.104	-2.43	0.652	6	-5.66	6

Particle toxicity – Oxidative stress (depletion of intracellular GSH)

None of the tested particles showed a reduction of total reduced GSH content, indicating no particle-induced oxidative stress. In fact, salt-laden glass (CaSO4_G) increased the relative abundance of reduced GSH. However, the the positive assay control *tert*-Butyl Hydrogen Peroxide (*t*BHP; 0.1-10 mM) either did not induce oxidative stress or was cytotoxic, in this particular batch of experiments, so the data could not be considered reliable.

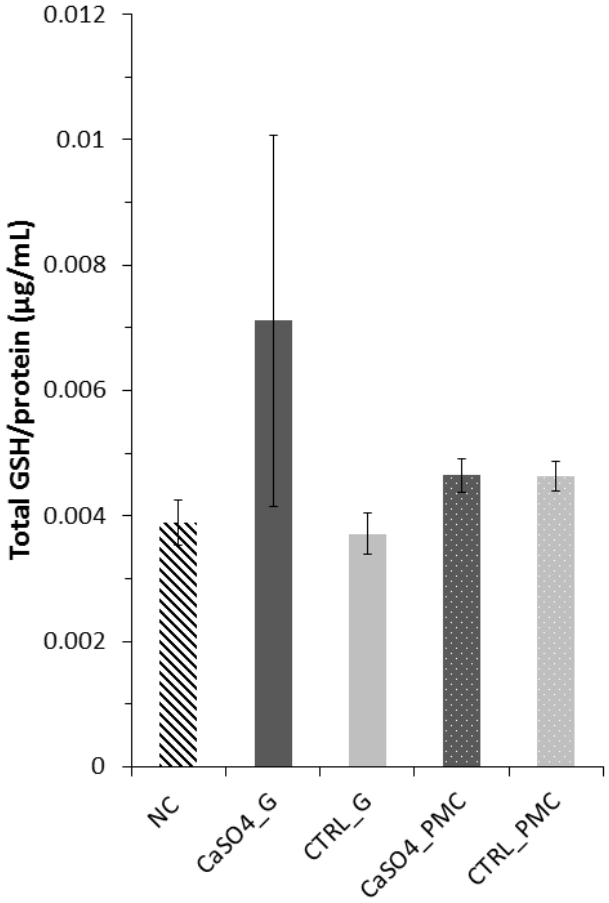


Figure A-5: Total glutathione (GSH) release in the culture medium following exposures to salt-laden glass (CaSO4_G) and pumice (CaSO4_PMC) and their respective controls (CTRL_G and CTRL_PMC). The positive assay control *tert*-Butyl Hydrogen Peroxide (*t*BHP; 0.1-10 mM) either did not induce oxidative stress or was cytotoxic (high LDH level). The negative control (NC) were untreated cells (cRPMI only). Data are presented as the mean ± standard error of the mean (n=3)

Particle toxicity – (pro-)inflammatory response (TNF-α and IL-1β)

Table A-6: TNF-α release in the culture medium following exposures to salt-laden glass (CaSO4_G) and pumice (CaSO4_PMC) and their respective controls (CTRL_G and CTRL_PMC).

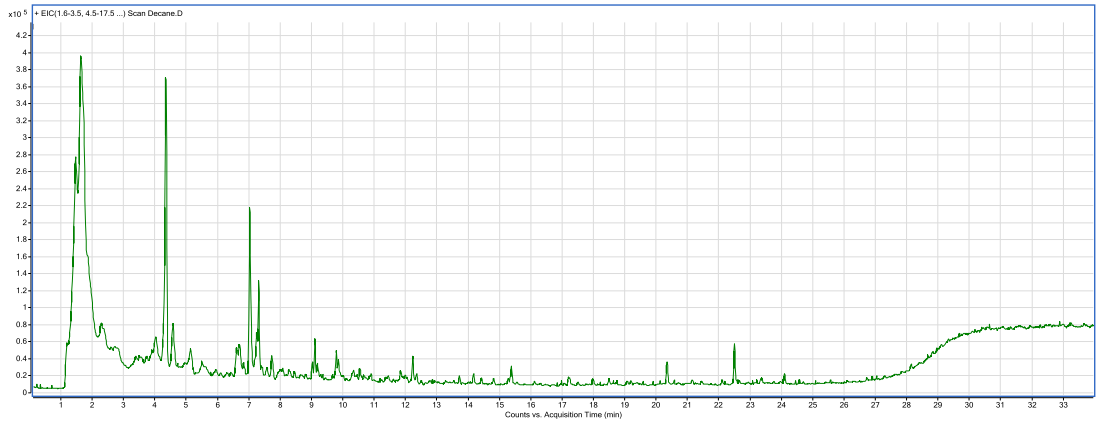
Sample	Exposure	Raw data (OD_{450})			Average concentration (ng/mL)	SD	SEM	DL (OD_{450})
CTRL_G	n=1	0.039	0.064	-	0.015	0.019	0.011	0.042
	n=2	0.070	0.069	0.078	0.009	0.001	0.001	0.037
	n=3	0.086	0.104	0.078	0.014	0.004	0.002	0.037
$CaSO_4$_G	n=1	0.075	0.075	0.069	0.010	0.001	0.001	0.037
	n=2	0.011	0.013	0.012	0.000	0.001	0.000	0.013
	n=3	0.011	0.012	0.012	0.000	0.000	0.000	0.013
CTRL_PMC	n=1	0.014	0.010	0.018	0.000	0.002	0.001	0.013
	n=2	0.008	0.009	0.007	0.000	0.001	0.000	0.013
	n=3	0.012	0.021	0.014	0.000	0.003	0.002	0.026
$CaSO_4$_PMC	n=1	0.016	0.012	0.011	0.000	0.001	0.001	0.013
	n=2	0.010	0.009	0.008	0.000	0.001	0.000	0.013
	n=3	0.022	0.029	0.018	0.000	0.004	0.002	0.026
NC	total n=4				0.001	0.009	0.004	
LPS	total n=6				0.681	0.371	0.185	

Table A-7: IL-1β release in the culture medium following exposures to salt-laden glass (CaSO4_G) and pumice (CaSO4_PMC) and their respective controls (CTRL_G and CTRL_PMC).

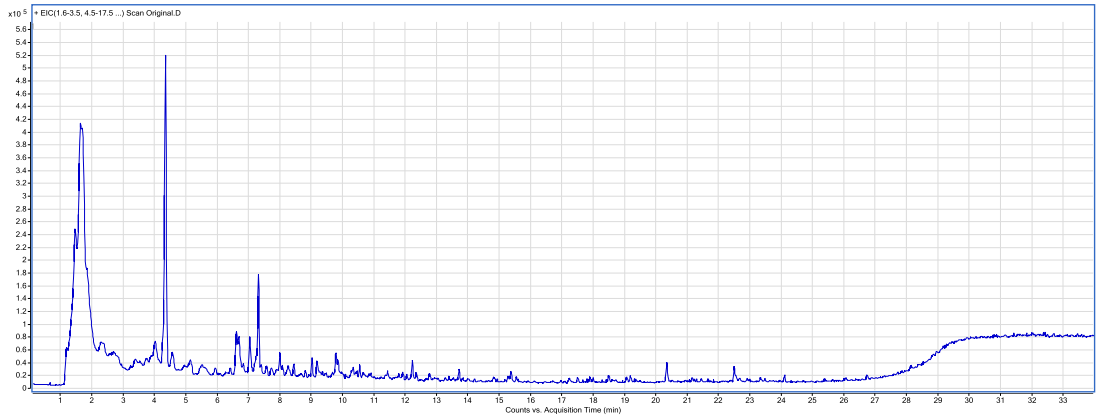
Sample	Exposure	Raw data (OD_{450})			Average concentration (ng/mL)	SD	SEM	DL (OD_{450})
CTRL_G	n=1	0.018	0.021		0.0000	0.000	0.000	0.038
	n=2	0.085	0.062	0.084	0.0000	0.001	0.001	0.037
	n=3	0.112	0.124	0.104	0.0001	0.001	0.000	0.037
$CaSO_4$_G	n=1	0.101	0.080	0.095	0.0000	0.001	0.001	0.037
	n=2	0.026	0.028	0.030	0.0003	0.000	0.000	0.017
	n=3	0.034	0.030	0.031	0.0007	0.000	0.000	0.017
CTRL_PMC	n=1	0.028	0.027	0.030	0.0003	0.000	0.000	0.017
	n=2	0.026	0.029	0.027	0.0002	0.000	0.000	0.017
	n=3	0.034	0.042	0.039	0.0016	0.000	0.000	0.014
$CaSO_4$_PMC	n=1	0.020	0.028	0.017	0.0000	0.001	0.000	0.017
	n=2	0.035	0.028	0.036	0.0008	0.001	0.000	0.017
	n=3	0.049	0.044	0.035	0.0020	0.000	0.000	0.014
NC	total n=5				*0.000*			
LPS	total n=5				0.112			

Py-GC-MS chromatograms

A) LT-HC treated ash



B) Untreated ash



Appendix 2

Publications

Tomašek et al. Particle and Fibre Toxicology (2016) 13:67
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Particle and Fibre Toxicology

RESEARCH Open Access

Combined exposure of diesel exhaust particles and respirable Soufrière Hills volcanic ash causes a (pro-)inflammatory response in an in vitro multicellular epithelial tissue barrier model

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Abstract

Background: There are justifiable health concerns regarding the potential adverse effects associated with human exposure to volcanic ash (VA) particles, especially when considering communities living in urban areas already exposed to heightened air pollution. The aim of this study was, therefore, to gain an imperative, first understanding of the biological impacts of respirable VA when exposed concomitantly with diesel particles.

Methods: A sophisticated in vitro 3D triple cell co-culture model of the human alveolar epithelial tissue barrier was exposed to either a single or repeated dose of dry respirable VA (deposited dose of 0.26 ± 0.09 or 0.89 ± 0.29 μg/cm^2, respectively) from Soufrière Hills volcano, Montserrat for a period of 24 h at the air-liquid interface (ALI). Subsequently, co-cultures were exposed to co-exposures of single or repeated VA and diesel exhaust particles (DEP; NIST SRM 2975; 0.02 mg/mL), a model urban pollutant, at the pseudo-ALI. The biological impact of each individual particle type was also analysed under these precise scenarios. The cytotoxic (LDH release), oxidative stress (depletion of intracellular GSH) and (pro-)inflammatory (TNF-α, IL-8 and IL-1β) responses were assessed after the particulate exposures. The impact of VA exposure upon cell morphology, as well as its interaction with the multicellular model, was visualised *via* confocal laser scanning microscopy (LSM) and scanning electron microscopy (SEM), respectively.

Results: The combination of respirable VA and DEP, in all scenarios, incited an heightened release of TNF-α and IL-8 as well as significant increases in IL-1β, when applied at sub-lethal doses to the co-culture compared to VA exposure alone. Notably, the augmented (pro-)inflammatory responses observed were not mediated by oxidative stress. LSM supported the quantitative assessment of cytotoxicity, with no changes in cell morphology within the barrier model evident. A direct interaction of the VA with all three cell types of the multicellular system was observed by SEM.

(Continued on next page)

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(Continued from previous page)

Conclusions: Combined exposure of respirable Soufrière Hills VA with DEP causes a (pro-)inflammatory effect in an advanced in vitro multicellular model of the epithelial airway barrier. This finding suggests that the combined exposure to volcanic and urban particulate matter should be further investigated in order to deduce the potential human health hazard, especially how it may influence the respiratory function of susceptible individuals (i.e. with pre-existing lung diseases) in the population.

Keywords: Volcanic ash, Diesel exhaust particles, In vitro, Particle co-exposures, Multicellular Human Epithelial Tissue Barrier System, Air-liquid Interface Exposures, (pro-)inflammatory cytokines/chemokines

Background

With nearly 10% of the world's population living near a historically active volcano [1], there is long-standing concern over the capacity of respirable-sized volcanic ash (VA) to cause acute and chronic respiratory health effects [2]. Substantial knowledge of the posed respiratory hazard, alongside extensive characterisation of the physicochemical properties of respirable VA, has been obtained in recent years [3, 4], leading to a better understanding of the structure-toxicity relationship [5]. However, with many volcanoes situated near large cities, VA is rarely inhaled in isolation; instead, VA is commonly exposed to the human population concomitantly with additional substances, notably anthropogenic pollution. A prime example of this is Mexico City, which was named by the United Nations as the world's most polluted city in 1992 [6] and sits just 70 km from the frequently-erupting Popocatepetl volcano.

Exposure to anthropogenic pollution is strongly associated with adverse health effects, predominantly pulmonary and cardiovascular diseases, as well as reduced respiratory health [7–12]. The human population resident in urban areas is particularly affected by high levels of anthropogenic particulate matter (PM) since vehicles are primary emitters of PM, with diesel exhaust particles (DEP) being the main constituent [13]. Yet, currently, limited understanding surrounds the human health hazard associated with the combined exposures (i.e. inhalation) that results from the addition of volcanic PM to the urban environment. Of particular importance is the consideration of how respirable VA may interact with DEP and how this may contribute, or not, to a heightened, potential respiratory hazard.

The aim of the present study, therefore, was to investigate the biological impact of a concomitant exposure to VA (Soufrière Hills volcano, Montserrat) and a standardised DEP sample (*National Institute of Standards and Technology's Standard Reference Material* (NIST SRM) 2975)) [14] for the first time, using an established, advanced multicellular in vitro model mimicking the human epithelial tissue barrier [15]. The basis for this project stemmed from the British Geological Survey's report to the UK Government on characteristics of a future large, effusive Icelandic eruption, which highlighted the urgent need to evaluate the role of mixing volcanic emissions with anthropogenic pollutants and whether this would affect the individual respiratory hazard of either particle independently [16]. Thus, the current study provides a landmark first assessment of these issues, the findings of which are highly relevant for volcanic health hazard management on a global scale.

Results

Particle characterisation

Particle size analysis of an isolated respirable fraction from the Soufrière Hills ash sample MVO12/7/03 showed that all particles were <10 μm. The sample consisted of 12.2, 41.5 and 72.5% volume of particles with sizes of <1, <2.5 and <4 μm, respectively (Additional file 1). Specific surface area, as determined by the Brunauer-Emmett-Teller (BET) [17] analysis with nitrogen adsorption, was 3.2 m^2/g.

Characteristics for the NIST SRM 2975 used were previously reported in [18]. Briefly, DEP exhibited a mean diameter of 1.62 μm (denoted by number distribution). DEP specific surface area was 91 m^2/g, as determined by BET with nitrogen adsorption.

Nebulisation of VA

A dry powder insufflator (DP-4, *Penn Century, USA*) was used to nebulise the respirable fraction of the VA for direct deposition onto the in vitro lung cell culture at the air-liquid interface (ALI). As this was the first study to administer VA at the ALI, an initial dose-dependent analysis of the VA deposition was conducted to determine cell-delivered dose, as well as its biological impact at these different doses. The cell-delivered dose was monitored using an integrated quartz crystal microbalance (QCM) and showed a concentration-dependent deposition of the VA sample (Fig. 1a). The average deposited doses were 0.13 ± 0.03, 0.21 ± 0.06, 0.26 ± 0.09 and 0.89 ± 0.29 $\mu g/cm^2$ (relative to an administered mass of 4, 6, 8 mg and a repeated administered mass of 8 mg, respectfully). The threshold limit for the QCM was 0.09 $\mu g/cm^2$. Scanning electron microscopy (SEM) imaging of the nebulised respirable ash sample (0.89 ± 0.29 $\mu g/cm^2$) revealed a heterogeneously dispersed deposition of ash particles (Fig. 1b).

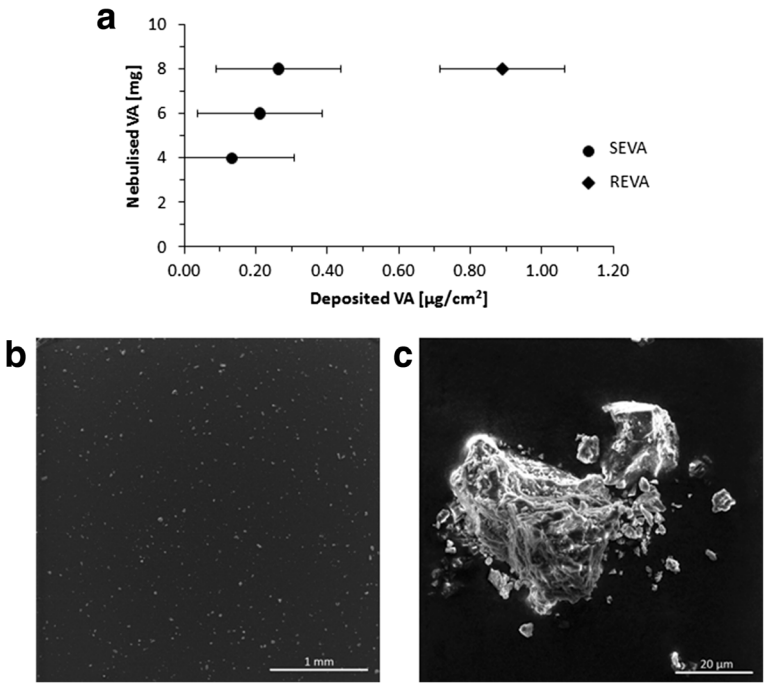

Fig. 1 Deposition of nebulized respirable fraction of volcanic ash. **a** Average mass deposition (μg/cm^2) of volcanic ash (VA) quantified using a quartz crystal microbalance (QCM), following nebulisation of dry respirable ash (*MVO12/7/03*) using a dry powder insufflator (DP-4*, Penn Century, USA*) under the following conditions: single exposure (SEVA) with 4 mg ($n = 14$), 6 mg ($n = 14$) or 8 mg ($n = 17$), as well as repeated exposure (REVA) to 8 mg (nebulised 3× within 15 min; $n = 9$). Data are presented as the mean ± standard error of the mean. Scanning electron micrographs of nebulized, uncoated ash sample (REVA), show **b** heterogeneous particle dispersion (WD: 5.53 mm, MAG: 97×) and **c** an inset of image (**b**) (WD: 7 mm, MAG: 3.80 k ×). Images were collected at 10 kV. Scale bars are 1 mm (**b**) and 20 μm (**c**)

Particle-cell exposures

Volcanic ash

An initial dose-dependent analysis of cytotoxicity, oxidative stress potential and (pro-)inflammatory response using administered VA masses of 4, 6 and 8 mg (Additional files 2 and 3) indicated all doses to be sub-lethal to the co-culture system. Due to the reliability, as well as a greater and effective deposition of the highest administered mass, it was subsequently used to assess the biological impact of VA as either a single exposure (SEVA; 0.26 ± 0.09 μg/cm^2) or repeated exposure (REVA; 0.89 ± 0.29 μg/cm^2) towards the in vitro triple cell co-culture model of the epithelial tissue barrier.

As determined *via* release of the cytosolic enzyme lactate dehydrogenase (LDH), no significant cytotoxicity ($p > 0.05$) was observed after 24 h following exposure to either SEVA or REVA compared to the negative control (defined as supplemented cell culture medium with no particle exposure) (Fig. 2g). The lack of any cytotoxicity associated with the SEVA and REVA exposures upon the cell cultures was qualitatively supported by the observation that no alteration to cellular morphology occurred, as visualised by laser scanning microscopy (LSM) (Fig. 2b and c). It was further observed, by LSM, that the epithelial layer was tightly bound together, forming a monolayer, with cells undergoing mitosis, suggestive of normal homeostasis (Fig. 2b and c). Further assessment of the biochemical impact of SEVA and REVA upon the triple cell co-culture showed no significant ($p > 0.05$) loss in total reduced glutathione (GSH), a key indicator of oxidative stress in vitro [19] (Fig. 3a). Similar, negative effects were also observed for the ability of either SEVA or REVA to elucidate a (pro-)inflammatory response, with no significant ($p > 0.05$) production of tumour necrosis factor-α (TNF-α) or interleukin-8 (IL-8) after 24 h exposure (Fig. 3b and c). It is important to note that, alongside these negative datasets, all positive controls used (i.e. *tert*-Butyl Hydrogen Peroxide (*t*BHP; GSH assay) and lipopolysaccharide (LPS; TNF-α and IL-8)) caused significant increases within the respective biological marker, indicating that the biological model used was responsive for all assay endpoints measured.

Diesel exhaust particles

Similar findings were observed following exposure of the co-culture to DEP alone, with no significant cytotoxicity (Fig. 2d and g) or changes ($p > 0.05$) to the oxidative stress status of cells observed (Fig. 3a). Importantly, the dose used of DEP was based upon the findings previously shown by Clift et al. [20], who undertook a dose-dependent analysis of the same DEP

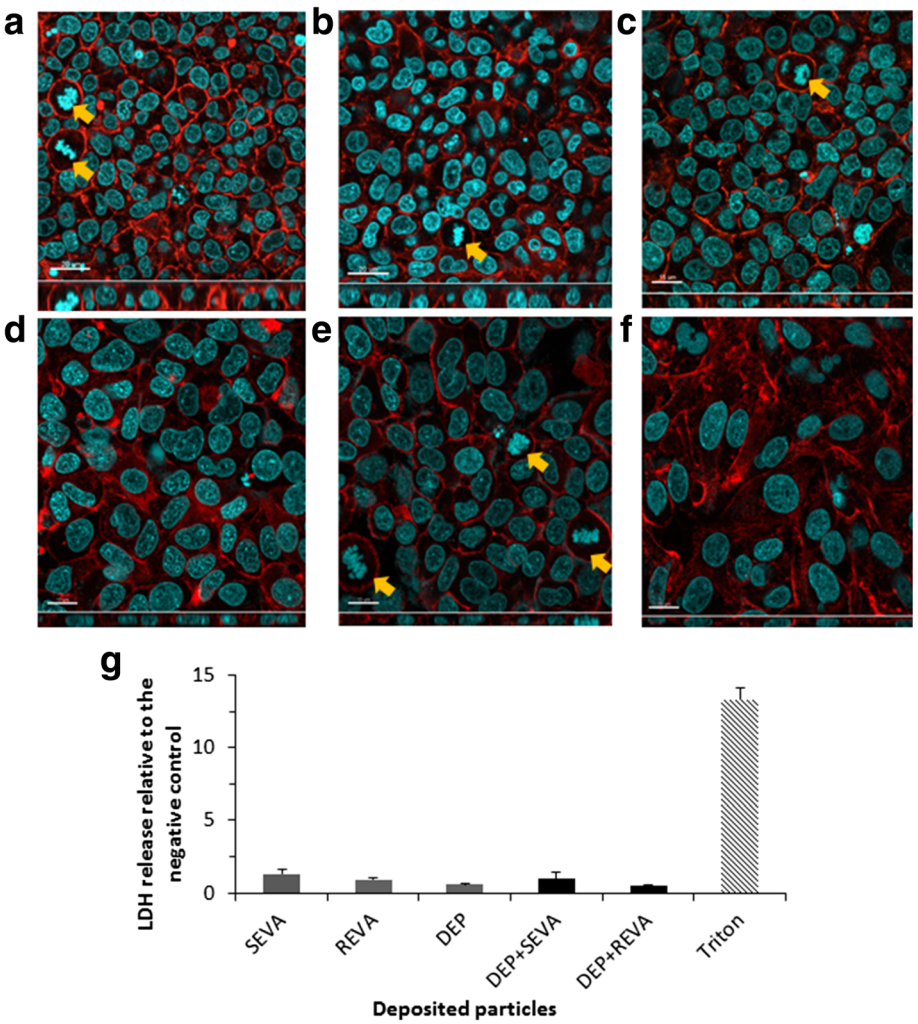

Fig. 2 Cell morphology and cytotoxicity of triple cell co-cultures exposed to volcanic ash and diesel exhaust particles. Confocal laser scanning microscopy (LSM) images show the complete triple cell co-culture (i.e. A549 type-II 'like' epithelial cell monolayer with human blood monocyte macrophages (MDM) and dendritic cells (MDDC) on the apical and basal sides, respectively) stained for F-actin cytoskeleton (*red*) and the nuclei (*blue*). **a** Control and cultures exposed to **b** 0.26 ± 0.09 μg/cm^2 of single exposure to volcanic ash (SEVA), **c** 0.89 ± 0.29 μg/cm^2, repeated exposure to volcanic ash (REVA), **d** diesel exhaust particles (DEP; 0.02 mg/mL), **e** diesel exhaust particles and 0.26 ± 0.09 μg/cm^2 of single exposure to volcanic ash (DEP + SEVA), and **f** diesel exhaust particles and 0.89 ± 0.29 μg/cm^2 of repeated exposure to volcanic ash (DEP + REVA). Yellow arrows indicate cells undergoing cell division. Scale bars are 20 μm (**a**-**b**) and 15 μm (**c**-**f**). Images were collected at magnification 63×. **g** Cytotoxicity determined by the release of lactate dehydrogenase (LDH) from the triple cell co-culture following exposure to SEVA, REVA, DEP, DEP + SEVA and DEP + REVA. Data are presented as fold increase relative to the negative control (supplemented cell culture medium only) ± standard error of the mean. Triton X-100 at 0.2% in phosphate buffered saline (PBS) acted as the positive assay control. LDH data shown are related to the following repetitions for each exposure: SEVA $n = 4$; REVA, DEP, DEP + SEVA and DEP + REVA $n = 3$; negative and positive controls $n = 8$

sample upon the same co-culture system. Again, the positive assay control, *t*BHP, showed a significant depletion of GSH in the co-culture system, confirming the observation that the array of sample exposures incited no oxidative stress. Despite these findings, the DEP only exposures to the in vitro multicellular system elicited a significant ($p < 0.05$) interleukin-1β (IL-1β) and non-significant ($p > 0.05$) TNF-α and IL-8 responses compared to the negative control (Fig. 3b and c).

Combined exposure of SEVA and REVA with DEP

Combined exposures to DEP and VA (DEP + SEVA, DEP + REVA) also resulted in no significant cytotoxicity (Fig. 2e, f and g) or changes ($p > 0.05$) to the oxidative stress status of cells (Fig. 3a). It was observed, however, that although the combined exposures did induce an heightened (pro-)inflammatory response in the co-cultures for TNF-α and IL-8 ($p > 0.05$), only a significant ($p < 0.05$) release of IL-1β, compared to the negative control, was noted (Fig. 3b–d).

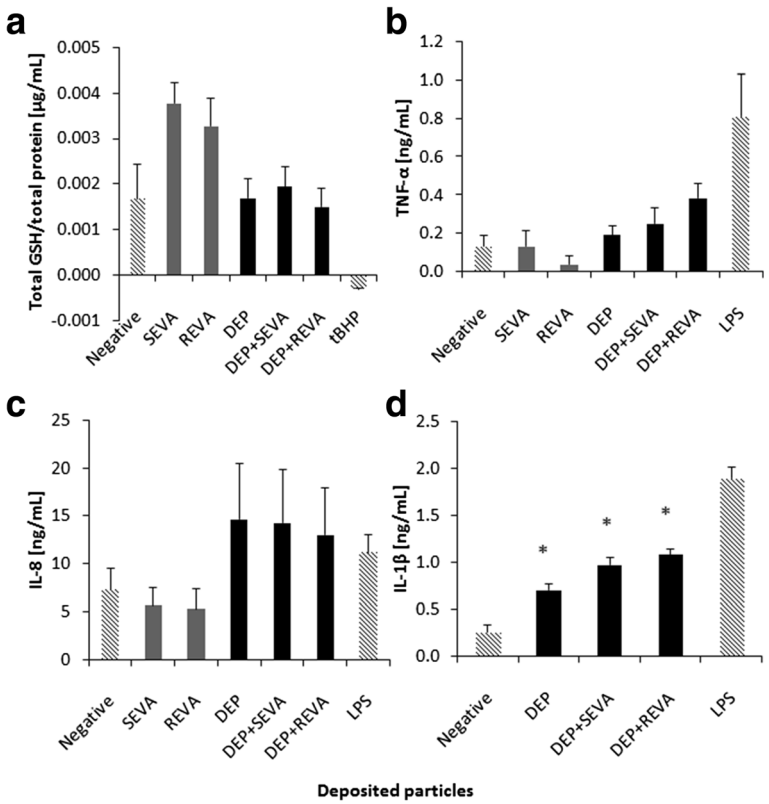

Fig. 3 Biochemical response of triple cell co-culture system following exposures to volcanic ash and diesel exhaust particles. **a** Total reduced glutathione (GSH), **b** tumour necrosis factor-α (TNF-α) release, **c** interleukin-8 (IL-8) release and **d** interleukin-1β (IL-1β) release of the triple cell co-culture model after exposure to 0.26 ± 0.09 μg/cm^2 of single exposure to volcanic ash (SEVA), 0.89 ± 0.29 μg/cm^2 of repeated exposure to volcanic ash (REVA), diesel exhaust particles (DEP; 0.02 mg/mL), co-exposure to diesel exhaust particles and 0.26 ± 0.09 μg/cm^2 of single exposure to volcanic ash (DEP + SEVA), and co-exposure to diesel exhaust particles and 0.89 ± 0.29 μg/cm^2 of repeated exposure to volcanic ash (DEP + REVA). The respective positive assay controls are *tert*-Butyl Hydrogen Peroxide (*t*BHP; 250 μL of 100 mM) and lipopolysaccharide (LPS; 100 μL of 1 μg/mL), added to the apical and basal compartment of the triple cell co-culture, respectively. The negative control was cell culture medium only. Data are presented as the mean ± standard error of the mean. Data shown are related to the following repetitions for each exposure: SEVA $n = 4$; REVA, DEP, DEP + SEVA and DEP + REVA $n = 3$; negative and positive controls $n = 8$. * indicates $p < 0.05$

Interaction of VA with triple cell co-cultures

SEM images of the upper surface of the triple cell co-culture exposed to dry VA at the ALI showed that VA was able to directly interact with the macrophage cache of the co-culture system. Interestingly, VA particles were also observed on the basal layer of the triple cell co-culture, elucidative of a potential interaction with the MDDC present in this region (Fig. 4).

Discussion

The purpose of this study was to gain a first understanding as to the potential hazard of a combined VA and DEP exposure to the respiratory system by using a state-of-the-art in vitro approach.

Particle concentrations

For VA, concentrations of 0.26 ± 0.09 μg/cm^2 (SEVA) and 0.89 ± 0.29 μg/cm^2 (REVA) were chosen. It is difficult to state how representative these concentrations are in relation to ambient air concentrations following a volcanic eruption, due to the lack of reliable in vivo dosimetry data available and the fact that airborne volcanic ash concentrations are highly dependent upon the distance of a person from the volcano and the dynamics of the eruption itself. In addition, concentrations of respirable ash will be raised during ashfall but, also, later, due to resuspension by wind and human activity in dry conditions, and will dramatically reduce following rain, making average and cumulative exposures difficult to constrain. Searl et al. [21] measured PM_{10} on Montserrat during a period of frequent ashfall (1997–1999) from the Soufrière Hills volcano and found daily mean concentrations ranging from 0.05 to 1 mg/m^3 when plentiful ash was in the environment, and 0.02–0.15 mg/m^3 when there was little ash. It is also important to note that personal exposure to volcanic ash is highly influenced by activities undertaken by individuals as well as the general dustiness of the environment; hence concentrations

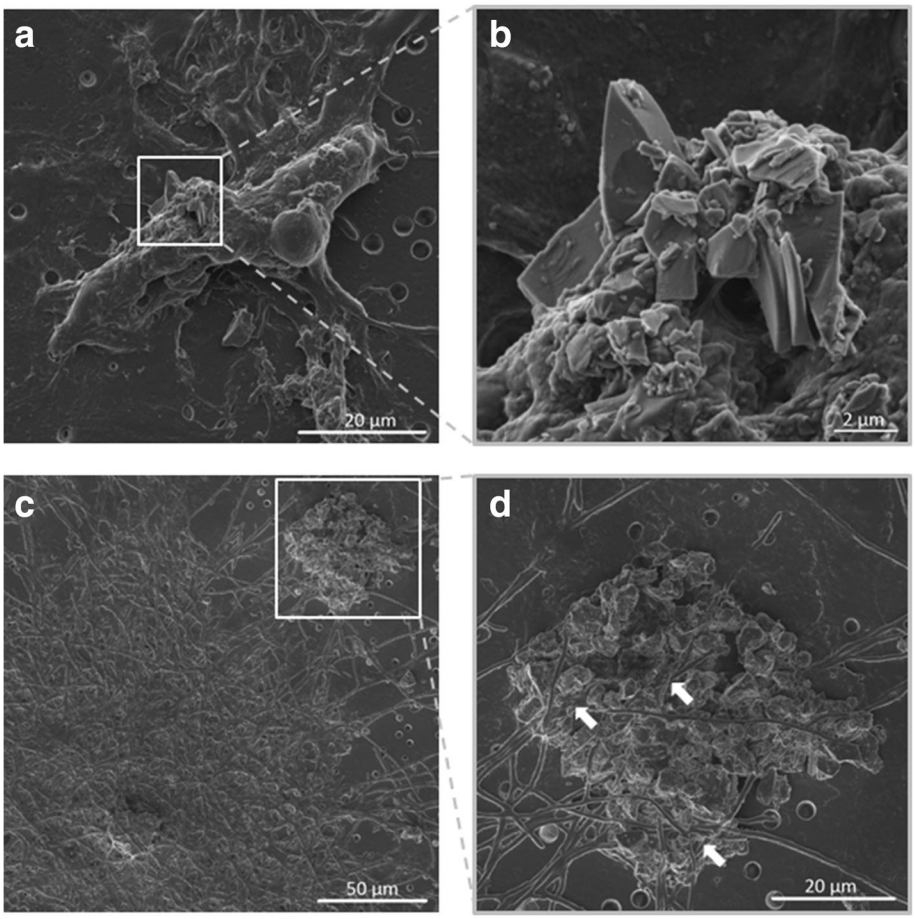

Fig. 4 Interaction of volcanic ash with the triple cell co-culture. Scanning electron micrographs of the triple cell co-culture membrane exposed to 0.26 ± 0.09 µg/cm^2 of single exposure to volcanic ash (SEVA), showing direct interaction of VA particles with the different cell types. **a** and **b** (inset of (**a**)) show a representative interaction of the human blood monocyte-derived macrophages (MDM) with VA, which appears to be engulfed by the MDM (**b**). Images (**c**) and **d** (inset of (**c**)) show a representative interaction of the human monocyte-derived dendritic cells (MDDC) with the VA particles, which are interacting with the pseudopodia of the MDDC (**d**; indicated with white arrows). Images were collected at 3 kV and 10 mm working distance. Scale bars are 20 µm (**a**), 2 µm (**b**), 50 µm (**c**) and 20 µm (**d**)

associated with activities such as cleaning, or clearing the roads, may be higher than background concentrations, especially considering children [21, 22]. However, the general community will have lower personal exposures than the ambient levels as people will protect themselves (e.g. staying indoors) during ashfall, overnight and at times of high resuspension.

Assuming a daily inhaled air volume for humans to be 25 m^3 and an alveolar lung surface area of *ca.* 100 m^2, which correspond to a healthy, moderately-active adult [23], and an alveolar deposition efficiency of about 10% [24], it can be estimated [25] that the doses used in this study correspond to airborne concentrations which would not be encountered over a 24 h exposure relative to dry conditions of the highest ash concentration areas during the most active phase of the Soufrière Hills eruption [21]. Thus, from a hazard assessment approach, the doses used in the present study can be considered as a 'worst-case', or a particle overload scenario relative to such an exposure period to humans.

For DEP, a pseudo-ALI approach was adopted as it was not possible to aerosolize the hydrophobic DEP with the dry powder insufflator device, due to the electrostatic nature of DEP in dry powder form. For this reason, VA and DEP could not be applied concomitantly to the cell cultures as a dry powder mixture. Instead, as previously described in [26], a volume of 100 µl of DEP suspension in supplemented medium at 0.02 mg/mL was added to the cells grown on a 4.2 cm^2 surface insert. Although these particles highly agglomerate, it was assumed that the majority of the DEP deposited on the cell surface at a dose of 0.5 µg/cm^2.

Notably, the present study is a first screening of potential adverse effects of combined exposure to VA and DEP, therefore a simple approach of comparing the effect of a combined exposure with the effects of individual particles

at comparable concentrations and exposure duration was used [27]. Importantly, the effects following exposures to individual particle types were considered, and single exposure data for DEP or VA are now compared to previous investigations using both in vitro and in vivo experiments.

Single particle exposure biological effects

In the present study, DEP, alone, caused no significant ($p > 0.05$) cytotoxicity or oxidative stress to the co-culture. Previous studies also conducted with the same multicellular model have shown that DEP do not enhance the release of LDH, although it has been found that they induce oxidative stress [20, 28, 29]. Variation among studies can be attributed to differences in the applied exposure method [30, 31] as previously, the same co-culture as used in the present study was exposed to the same type of DEP (NIST 2975), albeit applied *via* suspension (in supplemented cell culture medium) to the apical chamber of the insert [20, 29], thus initiating different particle-cell interaction kinetics. In the present study, as previously mentioned, a pseudo-ALI approach was used, as previously described [26]. Notably, the lack of cytotoxicity and oxidative stress observed in the present study is contrary to previous findings using monocultures of these cell types [32–36]. This difference can be associated with the cellular interplay exhibited by this multicellular model, where two immune cell types (macrophages and dendritic cells) can directly interact with each other at the epithelium during reactions to particulate antigens [37], thus further highlighting the advantages of using multicellular models. Overall, though, it should be taken into consideration that different cell cultures, DEP compositions, the preparation of particle suspension and doses used in different studies vary, which makes a direct comparison amongst these studies challenging. Nonetheless, in the current study, DEP did cause an increased release of measured (pro-)inflammatory markers (TNF-α, IL-8 and IL-1β) compared to the negative control (supplemented cell culture medium). These findings concur with previous observations of monoculture in vitro studies, which reported DEP to be highly (pro)-inflammatory [38, 39], as well as with studies using the same triple cell co-culture system [20].

Ash from the Soufrière Hills volcano has been extensively studied over the past two decades [40] and is well characterised for its physical and chemical properties, including the sample used here [3, 41, 42]. The biological impact of Soufrière Hills ash has also received increased attention during this time [43–46], particularly due to the substantial crystalline silica present in ash derived from collapses of the Soufrière Hills lava dome (a pile of extruded, viscous lava sitting within the crater) [42]. Previous results from monoculture in vitro studies performed with Soufrière Hills ash are variable, due to the various and numerous different experimental designs employed and endpoints considered as well as the large amount of natural variability amongst ash samples [2, 46]. Despite this variability, Soufrière Hills ash is generally considered to be non-cytotoxic and have a low oxidative potential, but has the capacity to incite a limited (pro-)inflammatory response. Previous cell-specific studies on macrophages (PMA-differentiated THP-1 monocytes) and A549 epithelial 'like' cells indicated minimal cytotoxicity (measured by LDH release) and GSH depletion following exposures [46], however the response of other antigen presenting cells to VA particles has been largely, to date, uncharacterised. Similar findings have also been noted from in vivo studies [44, 47–49]. In this study, assessment of the biological response from the triple cell co-cultures following VA exposures alone resulted in no significant ($p > 0.05$) cytotoxicity, changes in cellular morphology, oxidative stress or release of (pro-)inflammatory mediators (TNF-α and IL-8). Therefore, the findings of the initial dose-respose analysis of VA exposures alone are largely in congruence with previous research with Soufrière Hills ash [43, 46, 50].

As evident from the discussion above, all previous in vitro studies performed on VA have used monoculture cell models, where the cell cultures have been immersed in cell medium and VA particles added, already suspended i.e. a pre-mixed sample, in liquid medium. Whilst this approach is commonplace, it does not adequately reflect the physiological condition of a respiratory exposure to VA as particles do not settle in the lung already immersed in liquid (which may affect the surface reactivity of the VA sample). The nebulisation method of VA using the dry powder insufflator (*DP-4, Penn Century, USA*) has enabled, for the first time for in vitro studies, application of VA to cells in its pristine, dry state. This method represents a more realistic scenario in comparison to all previous studies which used suspended ash in cell medium. The method has also shown good reproducibility, and can, therefore, be considered as a suitable method for conducting realistic in vitro respiratory hazard assessment of VA in future research activities. Furthermore, it has recently been shown that multicellular models can be useful tools in determining the specific (pro-)inflammatory and oxidative stress effects of particles compared to monocultures; as these models additionally take into account intercellular signalling among cells as it would occur in vivo [20].

The observation, by SEM, that the macrophages directly interacted with VA was expected, due to their role in the clearance of foreign material, and previous studies have shown the capacity of macrophages to internalise VA [46, 51]. The interaction with epithelial cells is not completely unexpected either, due to the surface area that they cover in the alveolar epithelial airway barrier in

vitro system (insert membrane is 4.2 cm^2 with 1×10^6 epithelial cells seeded compared to 5×10^4 macrophages seeded in the co-culture) [15, 37]. It is worth noting that, to the best of the authors' knowledge, this was the first time that dendritic cells have been considered in terms of the biological impact of VA in vitro. The observed interaction of the VA with the MDDC can be hypothesized to occur either through (I) translocation of the VA particles *via* cell-cell interactions, as previously described for other particle types [37], (II) direct translocation through the pores of the micro-porous membrane insert (3 μm), or (III) deposition between the micro-porous membrane outer ridge and the side of the well of the six-well plate during the nebulisation process. Further research is therefore necessary to determine how VA becomes potentially available to interact, or not, with the dendritic cells of the co-culture system, such as through translocation studies previously performed with this 3D in vitro lung model [28, 52, 53], as well as to deduce what biological impact this interaction may potentially elucidate.

Co-exposure biological effects

At sub-lethal concentrations, as with the SEVA, REVA and DEP alone, the combined exposures (DEP + SEVA and DEP + REVA) showed no significant cytotoxicity in the triple cell co-culture. By working in this concentration range, it was therefore possible to study further, mechanistic effects. The impact of any particle type upon the respiratory system is commonly associated with an increased level of oxidative stress [54]. Yet, in the current study, it was observed that no significant differences in oxidative stress levels were evident in any of the combined particle exposures compared to the negative control. In light of these observations, DEP showed no deviation from the negative control but VA treatment increased the relative abundance of GSH, an observation previously attributed to increased production by macrophages (in monoculture) to cope with volcanic ash [46]. Therefore, comparatively, the effect of DEP on GSH levels is greater than the effect seen with VA alone, and the effects noted with the combined exposure scenario could be attributed to the DEP driving an oxidative stress environment in the cell cultures rather than the absence of any oxidative stress. However, to elucidate the underlying mechanisms controlling oxidative stress levels in this combined exposure, further research is needed. Despite the lack of any cytotoxic and limited oxidative stress response following VA and DEP combined exposures, it was found that a heightened (pro-)inflammatory response occurred following exposure to respirable VA and DEP when applied as a combined exposure. Focussing firstly on TNF-α release, although there was a greater effect with the DEP + SEVA exposure than that of the individual response for both SEVA and DEP, the greatest response was observed following the combined exposure of DEP + REVA. It is difficult to state though whether this effect can be described as synergistic in comparison to the two particle treatments alone [27]. Furthermore, the impact of the DEP can also be seen in the combined exposure IL-8 response, as the DEP, DEP + SEVA and DEP + REVA responses are all raised, albeit not significantly ($p > 0.05$), in comparison to the negative control. Given that the apparent driver of the (pro-)inflammatory signal is DEP present in the exposure, this was further assessed by analysing the (pro-)inflammatory cytokine IL-1β. Release of this marker was significantly higher ($p < 0.05$) in the DEP + REVA scenario compared to the negative control and to DEP alone. The observed higher response of (pro-)inflammatory markers following exposure to the DEP + REVA compared to the DEP + SEVA scenario can be attributed to the effect of the greater combined dose of particles delivered to the cell surface.

In summary, the current study provides a first insight into the biological effects of combined exposure to VA and an urban pollutant (DEP) and implies a potentially greater hazard of simultaneously inhaling both particle types. The observations indicate that combined exposure to VA and DEP induces a (pro-)inflammatory response in cells at the respiratory epithelial tissue barrier, but it is not yet clear whether this effect is directly driven by the individual particle-cell interactions, secondary toxicology mechanisms incited *via* the particles' physico-chemical characteristics, or through particle-particle interactions leading to the combined effect noted. It is known that increased release of (pro-)inflammatory mediators may augment, as well as prolong, inflammatory reactions and, if the exposure persists, can result in chronic inflammation [38]. Airway inflammation not only promotes the development of lung diseases, but it may increase the susceptibility to acute cardiovascular disease [55]. Thus, the significance of these findings lies in the potential effects on respiratory health that this combined exposure may elucidate. These initial results are being used to inform future, planned work investigating chemical interactions between particles and the particle/gas/volatile mixtures of complete vehicle exhaust [56, 57] as well as volcanic emissions.

Conclusion

The findings in the present study show that exposure to sub-lethal concentrations of VA with an urban pollutant (i.e. DEP) can promote a heightened and significantly increased (pro-)inflammatory response in vitro, absent of mediation by oxidative stress. The observed effects of the combined exposures are of further significance as, in some circumstances, they are greater than the response noted for DEP or VA, independently. It is envisaged that,

in the event of future eruptions, the findings of this study will serve for a better understanding of the potential respiratory risk posed by combined exposure to urban pollution and VA towards human health. These findings will provide the basis for further investigations into the mechanisms driving the heightened (pro-)inflammatory response, in order to deduce the specific human health hazard, as well as how it may influence the respiratory function of susceptible individuals (i.e. with pre-existing lung diseases) in the population.

Methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich (Switzerland), unless otherwise stated.

Particle samples

Volcanic ash

Source Ash from a dome-collapse ash-fall deposit of Soufrière Hills volcano, Montserrat (erupted and ash sample collected on 12 July 2003) was used (*MVO12/7/03*). The ash on Montserrat erupts into a very clean atmosphere (occasionally polluted by transfer of dust from the Sahara), so was chosen as a pristine example of ash which had no prior interaction with anthropogenic pollution. The bulk ash has previously been extensively characterised [3, 4, 41], and contains substantial quantities of respirable particles (cumulative volume % is 22.5 and 11.5 for <10 μm and <4 μm, respectively) [3] and is rich in crystalline silica (13.5 weight %) [4].

Sample preparation A respirable fraction of VA was isolated using a Sioutas Cascade Impactor (*SKC Inc., USA*) and Leland Legacy sample pump (*SKC Inc., USA*) attached to a gravitational separation chamber. VA was introduced into an airstream established by operating the pump at a constant flow rate of 5 L/min. Aerosolised particles then entered the separation chamber where particles above a theoretical spherical aerodynamic diameter of 5 μm sedimented in accordance with Stoke's law, calculated for a particle density of 1.0 g/cm^3 in the system. These parameters have been empirically observed to produce an appropriate respirable-sized fraction in this set up. Remaining airborne particles were then sampled by the Impactor, which was assembled without impaction stage filters to enable sample recovery. Size-fractionated samples were then collated for use in characterisation and toxicity assays. Separation of the respirable fraction from the sedimented ash was conducted upon the same subsample on three different occasions, and then combined in order to maximise the recovery of respirable material needed for the study.

Characterisation of VA respirable fraction

Particle size distribution analyses of isolated respirable samples were performed using a Coulter LS230 (*Coulter Corporation, USA*) in water without sonication. Data were analysed according to the Mie theory of light scattering [58]. Results are the mean of three consecutive runs of the sample. Runs were 60 s each.

Surface area was determined using samples dried overnight at 105 °C according to the BET method [17], and analysed by nitrogen adsorption measurements at -196.15 °C using a Gemini III 2375 surface area analyser (*Micromeritics Instrument Corporation, USA*). Results are the mean of three independent measurements of the sample.

Nebulisation of VA respirable fraction

Respirable VA was nebulised over the cells using a dry powder insufflator (Model DP-4; *PennCentury Inc., Philadelphia, USA*). The use of the dry powder insufflator was based upon the method previously described by Bihari et al. [59]. Briefly, the ash was loaded into a sample chamber and then pushed through the device by small pulses of air administered to the device using a 10 mL commercial air syringe. The ash was discharged as a cloud from the end of a delivery tube and, in this way, nebulised over the cell culture plate located below the delivery tube within a closed nebulisation chamber. The quantification of deposited material was monitored by a QCM (with a detection limit 90 ng/cm^2, AT-cut quartz, 5 MHz resonance frequency; *Stanford Research Systems, USA*) also located within the nebulisation chamber. Specifically, as material settles onto the QCM, the frequency of the crystal changes (ΔF). Calculated from the recorded frequency values before and after deposition of material, this ΔF value (Hz) is converted to deposited mass per area (μg/cm^2) as described in [31]. In addition, as previously highlighted in [26], the deposition pattern can possibly change across each well of the six-well culture plate used. Analysis of the data showed that there was no difference in the deposition pattern across each well of the six-well culture plates used (data not shown).

Scanning electron microscopy Nebulised respirable ash was imaged, uncoated, by a Mira3 LM (*Tescan, Czech Republic*) FE-SEM, using a secondary electron (SE) detector in order to visualise particle deposition and morphology.

Diesel exhaust particles

Source Standard diesel exhaust particulate (DEP; NIST SRM 2975) was used. The key characteristics of this standard sample have previously been reported [14, 59].

Sample preparation To produce a suspension of DEP, 1 mg of dry DEP was suspended in 1 mL cell culture medium RPMI 1640 (supplemented with 1% L-Glutamine, 1% Penicillin/Streptomycin and 10% fetal bovine serum). The pre-mixed solution was subsequently sonicated for 90 min at 37 kHz at 37 °C. This stock suspension of DEP was diluted with supplemented RPMI 1640 medium to a working concentration of 0.02 mg/mL.

Hazard assessment

Lung cell cultures

All in vitro exposure experiments in this study were conducted with an established 3D triple cell co-culture model of the human epithelial tissue barrier cultured at the ALI [15, 52, 60]. This system has previously been described in detail [61]. Briefly, the model consists of a layer of human alveolar type II-like epithelial cells (A549) with human monocyte-derived macrophages (MDM) on the apical side (upper chamber) and monocyte-derived dendritic cells (MDDC) on the basal side (lower chamber). A549 epithelial cells were cultured at a density of 1×10^6 cells per insert on BD Falcon cell culture inserts (high pore density PET membranes, 4.2 cm^2 growth area, 3.0 μm pore size; *Beckton Dickinson AG, Switzerland*).

Human blood monocytes were isolated from different, individual buffy coats received from the Swiss blood donation service (Bern, Switzerland) (i.e. different donor for each exposure), using $CD14^+$ MicroBeads as described previously [57]. Due to this, variations in the background among different sets of cell cultures were expected to occur. The cell culture densities of MDM and MDDC were 5×10^4 cells/insert and 25×10^4 cells/insert, respectively. Quantification of the cell-cell ratio for this co-culture system has previously been analysed and reported [37]. Co-cultures were incubated for 24 h under suspension conditions in order to allow cell-cell habituation. Subsequently cell culture medium was extracted from the apical layer to allow formation of the ALI over an extra 24 h period at 37 °C, 5% CO_2 prior to particle exposures.

Lung cell exposures

VA exposures In the approach used in this study, VA was administered as dry powder onto the upper surface of the co-culture at the ALI [37, 60] using a dry powder insufflator (Model DP-4; *PennCentury Inc., Philadelphia, USA*). Compared to the conventional particle suspension exposure, exposure at the ALI has been found to be a more sensitive in vitro exposure method, as it exhibits similar cellular responses at lower doses [30]. In addition, changes to the surface chemistry, morphology and size of the particles, which might affect the toxicological response of the system, are minimised.

As part of the initial dose dependent analysis to determine the optimal dose to use for VA in the combined exposure scenario, feed masses to the dry powder insufflator of 4, 6 and 8 mg of VA were used in a single dose exposure scenario. The corresponding dose that was deposited onto the cells was 0.13 ± 0.03, 0.21 ± 0.06 and 0.26 ± 0.09 μg/cm^2 for 4, 6, and 8 mg starting (feed) mass, respectively. Additionally, repeated exposure to the highest dose (8 mg) was used in order to increase particle mass deposition onto the cells, as 8 mg was the feed maximum per nebulisation. A mass of 8 mg was loaded into the dry powder insufflator and nebulised over the cells three times within a time period of ~15 min. The average mass deposited in the repeated exposure scenario was 0.89 ± 0.29 μg/cm^2.

DEP exposures DEP were used in a pseudo-ALI exposure experiment, as previously described [26]. Briefly, a total volume of 100 μl of DEP at 0.02 mg/mL suspended in supplemented medium was added to the apical compartment of the triple cell co-culture model at the ALI, grown on a 4.2 cm^2 surface trans-membrane insert. Upon assumption that the majority of the DEP would deposit homogeneously on the cell surface, the applied concentration would equate to a deposited concentration of 0.5 μg/cm^2. It is important to note that this methodology was used due to the fact that dry DEP were found to be unsuitable for nebulisation using the dry powder insufflator due to their electrostatic nature as a dry powder (data not shown).

VA and DEP combined exposures Directly after the exposure to DEP, the highest dose (chosen based upon the biological impact noted from the dose–response analysis and the efficiency of each dose deposited on the co-culture from the dry powder insufflator) of VA was applied, either as SEVA or REVA exposure, using the dry powder insufflator as previously described above in the section entitled '*Lung Cell Exposures; VA Exposures*'.

Post-exposure and sampling Each exposure was followed by a 24 h incubation period at 37 °C and 5% CO_2. After this, supernatants were collected and stored at either 4 °C or −80 °C until biochemical assays could be performed. In addition, insert membranes were fixed and prepared for immunofluorescent labelling or SEM microscopy, as described below.

Biochemical assays

Cytotoxicity

LDH Release Cytotoxicity, indicated by cell membrane damage, was determined by measuring the release of the intracellular enzyme lactate dehydrogenase (LDH) into the co-culture supernatant, assessed using an LDH

cytotoxicity detection kit (*Roche Applied Science, Mannheim, Germany*) according to the manufacturer's guidelines. The test was conducted in triplicate for each replication. The following repetitions for each exposure were conducted: SEVA $n = 4$; REVA, DEP, DEP + SEVA and DEP + REVA $n = 3$; negative and positive controls $n = 8$. Absorbance was determined at 490 nm after 10 min using a microplate reader (*Bio-Rad, Switzerland*), with a reference wavelength set at 630 nm. As a positive control, co-cultures were treated with 100 μl of 0.2% Triton X-100 in phosphate buffered saline (PBS) on the apical side and incubated for 24 h at 37 °C, 5% CO_2.

Cell morphology After the post-incubation period of 24 h, triple cell co-cultures were prepared for imaging *via* laser scanning microscopy (LSM). Cell membranes were fixed with 3% paraformaldehyde for 15 min at room temperature and then transferred to 0.1 M glycine in phosphate buffered saline (PBS) for 10 min. Samples were then washed x3 with PBS, and treated with 0.2% Triton X-100 for 15 min at room temperature to permeabilise the cell membrane for immunofluorescent staining. Subsequently, samples were stained with phalloidin-rhodamine (R-415; *Molecular Probes, Life Technologies Europe B.V., Zug, Switzerland*) in a 1:100 dilution to label the F-actin cytoskeleton, and with 1:100 dilution of 4′,6-diamidin-2-phenylindol (DAPI) at 1 μg/mL in 0.2% Triton X-100 + 1% BSA in PBS to highlight the cell nuclei. Visualisation of the samples was conducted with an inverted confocal LSM 710 (Axio Observer.Z1, *Carl Zeiss, Switzerland*) at a magnification of 63×. Representative images (z-stacks) were recorded at three independent fields of view for each sample (three independent samples were analysed ($n = 3$)) and were further processed using the 3D reconstruction software IMARIS (*Bitplane AG, Zurich, Switzerland*).

Oxidative stress

The total amount of reduced glutathione (GSH) in samples was quantified using a glutathione assay kit (*Cayman Chemical Company, Ann Arbor, Michigan, USA*) according to the manufacturer's instructions. The detected concentrations of GSH are reported relative to the concentrations of total protein of each corresponding sample (determined by the Pierce BCA Protein Assay kit (*Pierce Protein Research Products, Thermo Scientific, Rockford, Illionis, USA*)), according to the manufacturer's instructions. The negative control was cell culture medium only. As a positive control, co-cultures were treated with 250 μl of 100 mM tert-Butyl Hydrogen Peroxide (*t*BHP) on the apical side and incubated for 24 h at 37 °C, 5% CO_2. For each replication, analysis was conducted in triplicate. The following repetitions for each exposure were conducted: SEVA $n = 4$; REVA, DEP, DEP + SEVA and DEP + REVA $n = 3$; negative and positive controls $n = 8$.

Chemokine/cytokine release

The (pro-)inflammatory response was investigated by quantifying tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8) and interleukin-1β (IL-1β) release from the co-culture system into the basal cell culture well *via* enzyme-linked immunosorbent assays (ELISA DuoSet Development Kit, *R&D Systems, Minneapolis, Minnesota, USA*) according to the manufacturer's protocol. The concentrations were determined spectrophotometrically at 450 nm using a microplate reader (*Bio-Rad, Switzerland*). Lipopolysaccharide (LPS, from *E-coli* at 1 μg/mL) was applied at a volume of 1.2 mL in the bottom compartment of the co-cultures and served as the positive control for TNF-α, IL-8 and IL-1β induction. The negative control was cell culture medium only. Analyses were conducted in triplicate for each replicate. The following repetitions for each exposure were conducted: SEVA $n = 4$; REVA, DEP, DEP + SEVA and DEP + REVA $n = 3$; negative and positive controls $n = 8$.

VA:lung cell interactions in vitro

Scanning electron microscopy

Co-cultures exposed to SEVA were fixed with 3% paraformaldehyde for 15 min at room temperature and then sequentially washed with 20, 40 and 60% methanol for 5 min, 80% methanol for 3 min and washed 5 times with 100% methanol for 30 s. Samples were then dried in a vacuum desiccator over a 48 h period. Samples were then carbon coated and subsequently imaged with a Mira3 LM (*Tescan, Czech Republic*) FE-SEM, using an InBeam detector on a rotated stage (60°).

Data and statistical analysis

All data are presented as the mean ± standard error of the mean, deriving from three individual experiments ($n = 3$) unless otherwise stated. All statistical analyses were performed using SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0, *Armonk, NY, USA*). Statistical significance was deduced through the use of a one-way analysis of variance (ANOVA), based upon normal distribution of the datasets. Subsequent Tukey's *post hoc* tests were conducted to determine the specific statistical significance between the VA, DEP, DEP + SEVA and DEP + REVA exposures to the negative control (supplemented cell culture medium only). The alpha value was set at 0.05.

Additional files

Additional file 1: Particle size distribution of the isolated respirable fraction of Soufrière Hills volcanic ash. Determined by a Beckman Coulter LS230 PSD analyser (*Coulter Corporation, USA*). Data are the mean of $n = 3$. (TIF 30 kb)

Additional file 2: Cell morphology and cytotoxicity of the triple cell co-culture exposed to different single exposure doses of volcanic ash. Confocal laser scanning microscopy (LSM) images show the F-actin cytoskeleton (red) and the nuclei (blue) of (a) control and cultures exposed to (b) 0.13 μg/cm^2, (c) 0.21 μg/cm^2, and (d) 0.26 μg/cm^2 of respirable volcanic ash. Yellow arrows indicate cells undergoing cell division. Scale bars are 20 μm. Images were collected at magnification 63×. (e) Cytotoxicity as determined by the release of lactate dehydrogenase (LDH) from the triple cell co-culture following single exposure to 0.13 μg/cm^2, 0.21 μg/cm^2, and 0.26 μg/cm^2 of respirable volcanic ash. Data are presented as fold increase relative to the negative control (cell culture medium only) ± standard error of the mean. Triton X-100 at 0.2% in phosphate buffered saline (PBS) acted as the positive assay control. LDH data shown are related to the following repetitions for each exposure: SEVA $n = 4$; negative and positive controls $n = 8$. (TIF 524 kb)

Additional file 3: Biochemical response of the triple cell co-culture following exposures to different doses of volcanic ash. (a) Total reduced glutathione (GSH), (b) tumour necrosis factor-α (TNF-α) release, and (c) interleukin-8 (IL-8) release of the triple cell co-culture model after single exposure to 0.13 μg/cm^2, 0.21 μg/cm^2, and 0.26 μg/cm^2 of respirable volcanic ash. The respective positive assay controls are *tert*-Butyl Hydrogen Peroxide (*t*BHP; 250 μL of 100 mM) and lipopolysaccharide (LPS; 100 μL of 1 μg/mL), added to the apical and bottom compartment of the triple cell co-culture, respectively. The negative control was cell culture medium only. Data are presented as the mean ± standard error of the mean. Data shown are related to the following repetitions for each exposure: SEVA $n = 4$; negative and positive controls $n = 8$. (TIF 51 kb)

Abbreviations

ALI: Air-liquid interface; BET: Brunauer-Emmett-Teller; DEP: Diesel exhaust particles; ELISA: Enzyme-linked immunosorbent assay; GSH: Total reduced glutathione; IL-1β: Interleukin-1 beta; IL-8: Interleukin-8; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; LSM: Laser scanning microscopy; MDDC: Monocyte derived dendritic cells; MDM: Monocyte derived macrophages; NIST SRM: National Institute of Standards and Technology's Standard Reference Material; PBS: Phosphate buffered saline; PM: Particulate matter; QCM: Quartz crystal microbalance; REVA: Repeated exposure volcanic ash; SE: Secondary electron; SEM: Scanning electron microscopy; SEVA: Single exposure volcanic ash; *t*BHP: *tert*-Butyl Hydrogen Peroxide; TNF-α: Tumour necrosis factor - alpha; VA: Volcanic ash

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Availability of data and materials

All data and materials are specifically referenced within the manuscript. These are all openly available.

Authors' contributions

IT participated in the design of the study, carried out all biological - based experimentation and drafted the manuscript. CJH devised the project and was, as well as DED, AP-F, BR-R and MJDC, involved in the planning and technical advisory of the study. DED isolated the respirable fraction of VA and performed PSD and BET characterisation analysis. HB was involved in performing LSM imaging. CG was involved in SEM imaging. MJDC was the project leader; he was involved in planning the design of the study, has intellectually accompanied all experimental work, made substantial contributions to the analysis and interpretation of the data. CJH, DED, BR-R and MDJC have been involved in critically revising the manuscript for important intellectual content. All authors have confirmed approval of the final version of the submitted manuscript.

Competing interests

The authors declare no competing interests. The authors are entirely responsible for the data included in the present manuscript and furthermore for its written text.

Consent for publication

All authors have read and approved the manuscript for publication. There are no outstanding issues or requirements.

Ethics approval and consent to participate

Ethics regarding the use of human blood was considered under Swiss Federal Law. There were no requirements to obtain 'consent to participate', as it was not applicable for this study.

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Respiratory hazard assessment of combined exposure to complete gasoline exhaust and respirable volcanic ash in a multicellular human lung model at the air-liquid interface☆

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ABSTRACT

Communities resident in urban areas located near active volcanoes can experience volcanic ash exposures during, and following, an eruption, in addition to sustained exposures to high concentrations of anthropogenic air pollutants (*e.g.,* vehicle exhaust emissions). Inhalation of anthropogenic pollution is known to cause the onset of, or exacerbate, respiratory and cardiovascular diseases. It is further postulated similar exposure to volcanic ash can also affect such disease states. Understanding of the impact of combined exposure of volcanic ash and anthropogenic pollution to human health, however, remains limited.

The aim of this study was to assess the biological impact of combined exposure to respirable volcanic ash (from Soufrière Hills volcano (SHV), Montserrat and Chaitén volcano (ChV), Chile; representing different magmatic compositions and eruption styles) and freshly-generated complete exhaust from a gasoline vehicle. A multicellular human lung model (an epithelial cell-layer composed of A549 alveolar type II-like cells complemented with human blood monocyte-derived macrophages and dendritic cells cultured at the air-liquid interface) was exposed to diluted exhaust (1:10) continuously for 6 h, followed by immediate exposure to the ash as a dry powder ($0.54 \pm 0.19\ \mu g/cm^2$ and $0.39 \pm 0.09\ \mu g/cm^2$ for SHV and ChV ash, respectively). After an 18 h incubation, cells were exposed again for 6 h to diluted exhaust, and a final 18 h incubation (at 37 °C and 5% CO_2). Cell cultures were then assessed for cytotoxic, oxidative stress and (pro-)inflammatory responses.

Results indicate that, at all tested (sub-lethal) concentrations, co-exposures with both ash samples induced no significant expression of genes associated with oxidative stress (*HMOX1, NQO1*) or production of (pro-)inflammatory markers (IL-1β, IL-8, TNF-α) at the gene and protein levels. In summary, considering the employed experimental conditions, combined exposure of volcanic ash and gasoline vehicle exhaust has a limited short-term biological impact to an advanced lung cell *in vitro* model.

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Abbreviations

ALI	Air-liquid interface
CASP7	Caspase-7 gene
ChV	Chaitén volcano
CO	Carbon monoxide
CO_2	Carbon dioxide
cRPMI	Complete RPMI 1640 cell medium (supplemented with 1% L-Glutamine, 1% Penicillin/Streptomycin and 10% fetal bovine serum)
DEP	Diesel exhaust particles
FAS	FAS receptor gene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase gene
GDI	Gasoline direct injection
GE	Gasoline exhaust
HMOX1	Heme oxygenase 1 gene
IL-1β	Interleukin-1 beta
IL1B	Interleukin-1 beta gene
IL-8	Interleukin 8
IL8	Interleukin 8 gene
LDH	Lactate dehydrogenase
LOI	Loss on ignition
LSM	Laser scanning microscopy
MDDC	Monocyte-derived dendritic cells
MDM	Monocyte-derived macrophages
MDL	Method detection limit
NMHC	Non-methane hydrocarbons
NO_x	Nitrogen oxide(s)
NQO1	NAD(P)H dehydrogenase [quinone] 1 gene
PBS	Phosphate buffered saline
PET	Polyethylene terephthalate
PN	Particle number
PSD	Particle size distribution
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
SEM	Scanning electron microscopy
SHV	Soufrière Hills volcano
SiO_2	Silicon dioxide
THC	Total hydrocarbons
TNF-α	Tumor necrosis factor-alpha
TNFA	Tumor necrosis factor-alpha gene
VA	Volcanic ash
WLTC	Worldwide Light-duty Test Cycle
XRF	X-ray fluorescence

1. Introduction

Communities resident in urban areas located near active volcanoes can experience volcanic ash exposures during, and following, an eruption, in addition to sustained exposures to high concentrations of anthropogenic air pollutants (*e.g.,* vehicle exhaust emissions). Furthermore, ash can be transported over great distances (*e.g.,* as occurred during the 2010 Eyjafjallajökull eruption, Iceland (Gudmundsson et al., 2012)) and may reach distant urban areas, thereby having various impacts on human lives and livelihoods, including potential negative effects upon human health (Barsotti et al., 2010; Horwell and Baxter, 2006; Baxter & Horwell, 2015). Consequently, governments, public health agencies and scientific communities are voicing concerns about the potential health impacts from concomitant exposure to volcanic and anthropogenic emissions (Loughlin et al., 2012), especially whether this may pose a greater respiratory hazard than inhaling respirable volcanic ash and vehicle emissions separately. Hence, it is important to understand the potential hazard of concomitant exposure so that civil protection managers and health agencies can make informed decisions on whether to advise that citizens take action to protect themselves during periods of intense exposure to both urban pollution and volcanic ash (McDonald et al., 2017).

A recent first investigation, by the authors, into the potential effects of volcanic ash exposure combined with exhaust particulate (intending to simulate an urban environment) showed that concomitant exposure of cells to respirable volcanic ash and standardized diesel exhaust particles (DEP (NIST SRM 2975)) can promote a heightened (pro-)inflammatory response *in vitro* (Tomašek et al., 2016). However, the understanding of the respiratory hazard which may result from these combined exposures still remains limited, especially since this first study only considered DEP and not complete exhaust (*i.e.,* the additional gaseous component). Exhaust is a complex mixture that contains particles but, also, condensed and gaseous fractions. These phases can impact lung health (*e.g.,* Reed et al., 2008), but could also interact directly with the ash. This interaction may result in the adsorption of inorganic gases, such as CO_2, CO and NO_x, and volatile organic compounds (*e.g.,* linear and polycyclic aromatic hydrocarbons) onto volcanic ash, potentially altering the ash surface chemical properties and affecting its potential toxicity. Hence, the use of 'complete' exhaust is a critical next step in deducing the hazard posed to populations exposed to volcanic emissions and pollutants within the air.

Inhalation of urban particulate matter is known to cause the onset of, or exacerbate, respiratory and cardiovascular diseases (Pope et al., 2015; Peters et al., 1997; Seaton et al., 1995). Although diesel engines are generally viewed as greater contributors to engine-derived ambient particulate matter (US EPA, 2004), it has been found that vehicles with gasoline engines, which are still the most popular engine type in some European countries (ACEA, 2017), can also emit substantial quantities of soot-like ultrafine particles (diameter < 100 nm) under certain operating conditions (Mathis et al., 2005; Zhang and McMahon, 2012; Banerjee and Christian, 2017). Vehicles with gasoline direct injection (GDI) technology were found to release up to 10^{12} particles/km, exceeding those of current diesel vehicles equipped with filters (Platt et al., 2017; Muñoz et al., 2016; Zhang and McMahon, 2012; Mohr et al., 2006). The effects of emissions, including particulate and gaseous phases (*i.e.,* complete exhaust), produced from gasoline vehicles with various engine technologies have been studied in recent years and some toxic effects, such as oxidative stress and DNA damage in the lungs, have been reported in *in vitro* and *in vivo* studies (Bisig et al., 2015; Mauderly et al., 2014; Reed et al., 2008; McDonald et al., 2007; Lund et al., 2007).

It has been shown that exposure to respirable volcanic ash can exacerbate pre-existing respiratory diseases, such as asthma and bronchitis (Baxter et al., 1981, 1983), and suppress immune function (Monick et al., 2013). Even though there is a high variability in discrete results of *in vitro* and *in vivo* toxicology assessments for volcanic ash (reviewed by Baxter et al., 2014, Hillman et al., 2012, and Horwell and Baxter, 2006), a general view from the studies, to date, is that ash is a low toxicity particle, but various studies have shown that ash can provoke inflammatory reactions in the lungs and (pro-)inflammatory reactions *in vitro* (Damby et al., 2013, 2016, 2018; Horwell et al., 2013; Lee and Richards, 2004). Discerning the components of volcanic ash responsible for any observed toxicity

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has been difficult due to compositional variability amongst samples and eruptions. Possible mechanisms identified for ash toxicity involve the presence of reactive surface species, including, but not exclusively, iron, and the corresponding generation of reactive oxygen species (Horwell et al., 2003, 2007), and crystalline silica and its potential to activate the NLRP3 inflammasome (Damby et al., 2018; Baxter et al., 1999). The mechanisms resulting in the (pro-) inflammatory response *in vitro* following combined exposures to ash and DEP (Tomašek et al., 2016) are not yet clear, but may be driven by the individual particle-cell interactions, or possibly particle-particle interactions, which then interact with cells.

The aim of the present study was to assess the biological impact of combined exposure to cells of respirable volcanic ash and complete vehicle exhaust. A sophisticated *in vitro* approach, as also used in our previous study (Tomašek et al., 2016), provides a valuable first assessment of potential adverse impacts of such exposures, especially due to a lack of epidemiological studies that consider health effects of ashfall in heavily polluted urban areas.

Volcanic ash samples, from Soufrière Hills volcano, Montserrat, and Chaitén volcano, Chile, were used (to represent different magmatic compositions and eruption styles) in combination with freshly-generated complete gasoline exhaust from a GDI vehicle (containing the particulate, condensed and gaseous fractions). This is the first time that a real, complete exhaust has been used to study combined exposures with volcanic ash. Furthermore, this investigation is the first to evaluate and report on whether the toxicity of either volcanic ash or complete gasoline exhaust are altered by co-exposures, as well as whether the ash (magmatic) composition could influence the outcome of combined exposures *in vitro*.

As with our previous study, a multicellular *in vitro* human lung model, composed of epithelial lung cells (A549 alveolar type II-like cells) and two immune cell types (human blood monocyte-derived macrophages and dendritic cells) (Rothen-Rutishauser et al., 2005, 2008; Blank et al., 2006) cultured at the air-liquid interface (ALI) (Blank et al., 2007) was used. This well-established model has been proven to be suitable for various exposures at the ALI (Fytianos et al., 2016; Müller et al., 2011), thus reflecting, in part, the realistic physiological conditions of a respiratory exposure. Further, human epithelial airway barrier models have previously accompanied hazard assessment studies of products of different engines and/or fuels using a sophisticated, well-characterised exhaust exposure system (Bisig et al., 2015, 2016; Steiner et al., 2013a,b; Müller et al., 2012, 2011, 2010). Here, the multicellular model was directly exposed to gasoline emissions, followed by the addition of respirable volcanic ash and a second gasoline emissions exposure. Subsequent analyses of the cell cultures for cytotoxicity, oxidative stress and (pro-)inflammatory response were undertaken.

2. Materials and methods

2.1. Cell cultures

In vitro experiments were performed using an established multicellular lung model composed of three cell types mimicking the human alveolar epithelial tissue barrier as previously described (Rothen-Rutishauser et al., 2005, 2008; Blank et al., 2007). Briefly, the model consists of a layer of human alveolar type II-like epithelial cells (A549) cultured on polyethylene terephthalate (PET) membrane 6-well inserts (4.2 cm^2 growth area, 3.0 μm pore size; BD Falcon™ Cell Culture Inserts, BD Biosciences, USA) at an initial density of 23.8 x 10^4 cells/cm^2. These were grown for 5 days prior to the addition of immune cells to form a co-culture. Human blood monocytes were isolated from buffy coats provided by the Transfusion Blood Bank (*Blutspendedienst SRK Bern AG*, Switzerland) as described previously (Lehmann et al., 2010) with the adaptation of using $CD14^+$ magnetic beads (CD14 MicroBeads, *Miltenyi Biotec*, Germany) (Steiner et al., 2012). The monocyte-derived macrophages (MDM) and dendritic cells (MDDC) were added on the apical (1.2 x 10^4 cell/cm^2) and the basal (6.0 x 10^4 cell/cm^2) side of the insert with a A549 layer on the upper side of the insert, respectively. After 24 h incubation under submerged conditions, in complete RPMI 1640 cell medium (cRPMI; *Sigma-Aldrich*, Switzerland; supplemented with 1% L-Glutamine, 1% Penicillin/Streptomycin and 10% fetal bovine serum), the triple-cell co-culture was transferred to the ALI (the cRPMI only present on the basal side of the insert) to habituate for a period of 24 h prior to exposures.

2.2. Volcanic ash sources, preparation and characterisation

Every explosive volcanic eruption generates ash, but particle characteristics (*e.g.*, surface area and reactivity, composition, particle size, leachable elements) and, potentially, toxicity will vary according to the magma composition and eruption style as well as post-eruptive factors such as distance from vent (of deposition), weathering, etc. Two volcanic ash samples were used in this study, to represent different magmatic compositions (andesite and rhyolite; see Section 3.1) and eruption styles. Both samples were chosen because they were erupted, transported and deposited into relatively-clean atmospheres, away from major sources of pollution. Neither was rained on prior to sampling. In addition, both samples have been well characterised for their physical and chemical properties in previous studies (Horwell et al., 2010, 2013; Horwell, 2007). The major elemental oxide composition of bulk samples was determined by X-ray fluorescence (XRF) (Axios-Advanced PW4400 XRF spectrometer, *PANalytical*, The Netherlands) on fused beads prepared from ignited ash powders mixed with a fluxing agent in a 1:5 ratio. Compositional data were recalculated to account for loss on ignition (LOI) (the weight difference between unignited and ignited powders), which also provided confirmation of sample freshness with regards to contamination (*e.g.*, with organic material).

The first ash sample, from the Soufrière Hills volcano (SHV), Montserrat was generated in a dome-collapse event on 12 July 2003 and was collected 4 km from the vent on the day of eruption. The bulk sample's physicochemical characterisation can be found in previous literature under different sample codes, as follows: *Soufrière Hills, Montserrat '03* in Horwell et al. (2007), Soufrière Hills, Montserrat 2003 in Horwell (2007), *Mon12/7/03* in Horwell et al. (2010) and *MBA12/7/03* in Horwell et al. (2013). Briefly, the ash is rich in crystalline silica (~12 wt% of the bulk ash is cristobalite), potentially the most pathogenic of the minerals found in volcanic ash (Baxter et al., 1999), and is considered fine grained for ash, with ~11.5 vol% of the bulk sample being sub-4 μm diameter (Horwell, 2007). This sample was used in the previous study investigating co-exposures with DEP in the same *in vitro* multicellular model (as *MVO12/7/03* in Tomašek et al., 2016).

The second sample was obtained from Chaitén volcano (ChV), in Patagonia, Chile and was deposited from an explosive eruption which occurred on 2 May 2008. The sample was collected 80 km away from the source, in the Patagonian Argentine province of Chubut, on 8 May 2008 (Horwell et al., 2010). This sample contains substantially less crystalline silica (~3 wt% of the bulk ash is cristobalite) than the SHV sample, but is similarly fine-grained, with ~12 vol% sub-4 μm material (as *Chai_03* in Horwell et al., 2010).

A Sioutas Cascade Impactor (*SKC Inc.*, USA) and Leland Legacy sample pump (*SKC Inc.*, USA) attached to a gravitational separation chamber were used to isolate a biologically relevant 'respirable' sub-sample of the bulk ash for use in the *in vitro* exposure model, as previously described (Tomašek et al., 2016). Briefly, bulk ash was introduced into the separation chamber by an airstream

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established by the pump at a constant flow rate of 5 L/min. Particles above a theoretical spherical aerodynamic diameter of 5 μm sedimented while the remaining, smaller particles were sampled by the impactor. Size-fractionated samples were recovered and combined for use in characterisation and toxicity assays.

Particle size distributions (PSD) of isolated respirable samples were determined by Mie theory with a laser particle analyser (Beckman-Coulter LS 13 320; *Coulter Corporation,* USA) in water with sonication, with a refractive index set to 1.63 and an absorption coefficient of 0.1 (after Horwell, 2007). Results are presented as the average of three consecutive measurements of the sample. Scanning electron microscopy (SEM) was used to observe particle morphology. Particles were mounted on polycarbonate discs, coated with 30 nm of gold/palladium and imaged on a Hitachi SU-70 FEG SEM (*Hitachi, Ltd.*, Japan) using the secondary electron detector.

2.3. Volcanic ash exposure system

In order to deduce the individual and combined response from the co-culture, respirable ash samples were nebulized as dry powder directly over the cell cultures at the ALI using a dry powder insufflator (Model DP-4; *PennCentury Inc.,* USA). The dry powder insufflator was found suitable for this application in the previous study (Tomašek et al., 2016), where its efficiency in ash administration to cells was evaluated, representing a more realistic approach when compared to studies using pre-suspended ash in submerged cell culture conditions.

The exposure setup was used as described previously (Tomašek et al., 2016). Briefly, the ash was loaded into the sample chamber and then pushed through the device by small pulses of air administered using a 10 mL commercial syringe. The ash was discharged as a cloud over the cell culture plate located below the delivery tube within a closed nebulisation chamber. Two cell culture inserts from the same set (exposed beforehand to the exhaust or filtered air, see section 2.6) were exposed simultaneously to a single ash sample (SHV or ChV). The chamber was made of polystyrene and covered with aluminium foil on the inside to avoid particles being electrostatically attracted and sticking to the chamber walls. The quantification of deposited material was monitored by a quartz crystal microbalance (QCM; *Stanford Research Systems,* USA), also placed within the nebulisation chamber next to the wells, thereby allowing for a reliable estimation of the deposited mass. Calculated from the recorded frequency values before and after deposition of material, the ΔF value (Hz) is converted to deposited mass per area (μg/cm^2) (Lenz et al., 2009).

2.4. Vehicle exhaust exposure system

A flex-fuel GDI vehicle with a three-way catalyst was driven on a chassis dynamometer with standard market gasoline (RON 95) and lubrication oil. A dynamic, worldwide light-duty test cycle (WLTC) (UNECE, 2016), representing transient driving in urban, extra-urban, highway and motorway conditions, was driven and repeated for 6 h per day of exposures (10 cycles). The WLTC is the official driving cycle from September 2017 onwards used by the European Union for new vehicle registration (Euro6).

The exhaust exposure experiments were performed at the exhaust gas control station of the Bern University of Applied Sciences in Nidau, Switzerland, as previously described (Bisig et al., 2015; Müller et al., 2010, 2011; Morin et al., 2008). Briefly, the exhaust was diluted 1:10 in filtered air, based on previous work (Steiner et al., 2013a, 2013b) and to enable comparison with previous gasoline exhaust studies (Bisig et al., 2015, 2016), where it was noted that it represents a highly-polluted site (*i.e.,* a high dose exposure).

The diluted exhaust was pumped through the cell culture exposure chamber with a constant flow of 2 L/min. In the chamber, the exhaust emissions pass above the cell culture plates and diffuse onto the cell cultures. Simultaneously, in a reference chamber, filtered ambient air supplied under identical conditions served as the negative control. The conditions in both chambers were controlled at 37 °C, 85% relative humidity and 5% CO_2.

2.5. Exhaust characterisation

Characterisation of the exhaust was performed in parallel to the exposure experiments, yielding detailed information on the emission sample that the cells were exposed to. Measurements were taken during the initial exposures (n = 2) and repeated exposures (n = 2) (each 10 WLTC cycles) resulting in 4 distinct datasets. The particle number (PN) was measured in the 1:10 diluted exhaust using an engine exhaust condensation particle counter (Model 3790, *TSI Inc.*, USA). The concentrations of carbon monoxide (CO), carbon dioxide (CO_2), total gaseous hydrocarbons (THC), non-methane hydrocarbons (NMHC) and nitrogen oxides (NO_x) were measured using a Horiba MEXA-9400H (*Horiba*, Japan) exhaust gas measuring system in a constant volume sampling tunnel (Horiba CVS-9500 T, *Horiba,* Japan). CO_2 was added to reach 5% for optimal buffering capacity in the cell culture medium. The concentration was controlled with two sensors, right before and after the chamber, and adjusted with a flowmeter, if needed.

2.6. Cell culture exposures

The two-day cell exposure scenario (Fig. 1) was designed to simulate a real-life situation when volcanic ash is introduced to the urban environment (where people are continuously exposed to urban pollution; *e.g.,* gasoline vehicle emissions), resulting in pollutants being concomitantly inhaled. Two sampling time-points were chosen, 24 h and 48 h, to enable observation of the effects after one day of co-exposure (as in the previous study by Tomašek et al., 2016) and, in addition, to account for possible effects following another, repeated exposure to gasoline exhaust.

Daily experimental exposure to diluted (1:10) gasoline exhaust for 6 h was chosen according to earlier studies (Bisig et al., 2015, 2016; Steiner et al., 2013a; Steiner et al., 2012). It is difficult to assess whether this represents real life exposures, but could represent exposure of an urban outdoor worker. The cell-delivered volcanic ash doses fall within the range of the lowest and highest doses used in the previous study (Tomašek et al., 2016). In order to achieve equivalent mass exposure for both ash samples, the QCM was monitored during the exposures until a target dose between 0.4 and 0.5 μg/cm^2 was reached. There is a lack of dosimetry data for inhalation of ash, or exposure data on ambient air concentrations following volcanic eruptions, making average exposures difficult to constrain and apply *in vitro*, but Tomašek et al. (2016) determined their dose range to be a worst-case scenario. Therefore, these doses may not be realistic for personal exposure and could be considered as a particle over-load relative to a real-life exposure. Overall, the chosen exposure scenario may be considered as a short-term, high-level exposure to both pollutants, individually and when combined. Even though the doses used likely deviate from realistic inhalation exposure, the assessment of cellular responses herein can be seen as a valuable screening of possible (adverse) effects that this specific type of combined exposure may incite which has not previously been considered or investigated.

The multicellular lung model was exposed at the ALI to diluted exhaust (1:10) or filtered air (reference chamber) continuously for

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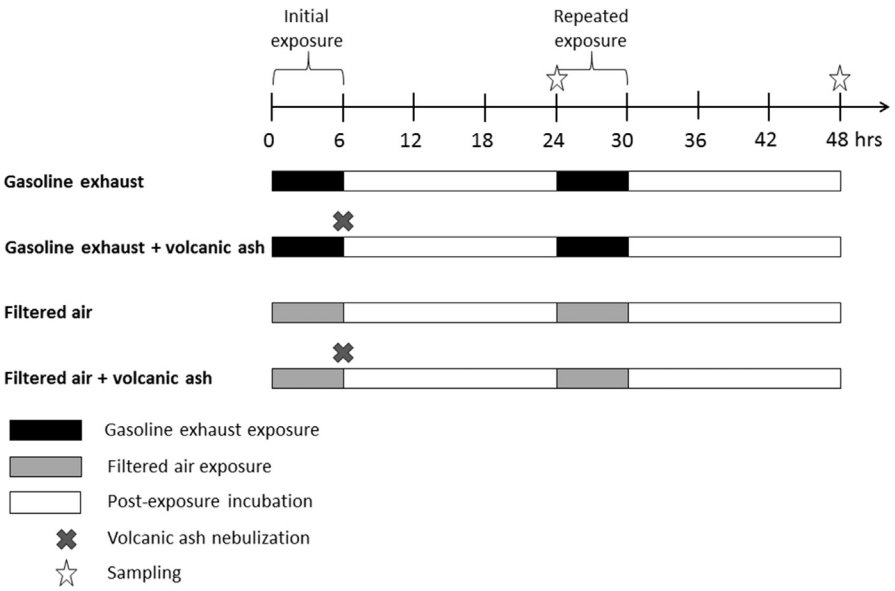

Fig. 1. Schematic of the cell culture exposures at the ALI in the present study. Culture supernatant was sampled at the 24 h time-point, and both the supernatant and insert membranes were sampled at the 48 h time-point. Cell-cultures were maintained under air-liquid interface (ALI) conditions throughout the entire exposures (initial exhaust, volcanic ash, repeated exhaust).

6 h (*i.e.,* the initial exposure), followed by immediate exposure to respirable volcanic ash as described in section 2.3, and then incubated (at 37 °C and 5% CO_2) for 18 h, maintaining the ALI conditions. Subsequently, the supernatants (*i.e.,* cell culture medium) were collected (24 h time-point) from the basal side of the insert and replaced with fresh cell medium. Cells were exposed again for 6 h to diluted exhaust or filtered air (*i.e.,* the repeated exposure), followed by a final 18 h incubation, maintained under ALI conditions. The supernatants were then collected (48 h time-point) (Fig. 1). Each 48-h exposure scenario (exhaust, ash, repeat exhaust) was conducted with two different sets of the multicellular lung model (*i.e.,* with cells from different passage numbers and monocyte isolations), each exposed separately to volcanic ash after the initial exposures, resulting in two replicates (n = 2) per exposure scenario. Two exposure scenarios were conducted over a 4-day period, resulting in 4 experimental replicates in total (n = 4 per ash sample). All data are presented relative to the filtered air (reference) cultures, however, we also included an untreated control (kept in the incubator) to assess the influence of the exposure protocol on cell response (see supplementary information). Collected supernatants were stored at either 4 °C or −80 °C prior to biochemical assays (section 2.7). Insert membranes were split and one half of each replicate's membrane was used for gene expression analysis (section 2.7.4) whilst the other half was fixed and prepared for fluorescent labelling, as described in section 2.7.1.

2.7. Cellular assays and analysis

Cell culture supernatants collected at 24 and 48 h time-points (Fig. 1) were analysed for key/relevant bio-markers of pulmonary-related toxicity *in vitro* (Donaldson et al., 2005). All analyses were performed according to established, standardized protocols as described below.

2.7.1. Cell morphology

Cell membranes collected at the 48 h time-point were fixed with 4% paraformaldehyde (15 min, room temperature), washed and stored in phosphate buffered saline (PBS). Subsequently, they were permeabilised with 0.2% Triton X-100 *(Sigma-Aldrich*, Germany) in PBS (15–30 min, room temperature). Samples were then stained with the F-actin stain Phalloidin-Rhodamine (*Thermo Fisher Scientific Inc.,* USA) and the nuclei stain 4',6-diamidin-2-phenylindole (DAPI; *Sigma-Aldrich,* Germany) diluted 1:50 and 1:100 in 0.1% BSA in PBS (1–2 h, room temperature), respectively, and then mounted with Glycergel (*DAKO Schweiz AG,* Switzerland) on microscope slides.

Images of the cell membranes were acquired via confocal laser scanning microscopy (LSM; Zeiss 710 Confocal Microscope, *Carl Zeiss,* Switzerland) using a 63x/1.4 NA oil immersion lens. Representative images were processed using the public domain image analysis software ImageJ (http://rsb.info.nih.gov/ij).

2.7.2. Lactate dehydrogenase release

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) into the co-culture supernatant, at both 24 h and 48 h time-points, using a LDH Cytotoxicity Detection Kit (*Roche Applied Science,* Germany) according to the manufacturer's protocol. The test was conducted in duplicate for each experimental replication. Absorbance was determined at 490 nm using a microplate reader (*Bio-Rad,* Switzerland), with a reference wavelength set at 630 nm. The positive assay control was 100 μL of 0.2% Triton X-100 in PBS, applied for 24 h on the apical side of the cell culture insert.

2.7.3. Quantification of (pro-)inflammatory response

The quantity of tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8) and interleukin-1 beta (IL-1β) secreted into the culture medium was assessed in the co-culture supernatants collected at 24 h and 48 h time-points by enzyme-linked immunosorbent assay (ELISA DuoSet Development Kit, *R&D Systems,* USA) according to the manufacturer's protocol. The concentrations were determined spectrophotometrically at 450 nm using a microplate reader (*Bio-Rad,* Switzerland). Analyses were conducted in duplicate for each repetition. The positive assay control was lipopolysaccharide (LPS, from *E-coli* 055:B5 strain (*(Sigma-Aldrich*, Germany), 1 μg/mL) applied as 1.2 mL solution in cRPMI in the bottom compartment of the cell culture insert for 24 h.

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2.7.4. Gene expression analysis

Quantification of gene expression at the transcriptional level was performed by real-time reverse-transcription polymerase chain reaction (real-time RT-PCR), as previously described (Bisig et al., 2015). Cell culture membranes sampled at the 48 h time-point were stored in the ribonucleic acid (RNA) protect buffer (RNAprotect® Cell Reagent, *Qiagen,* Germany; diluted in PBS 1:4 (v/v)) prior to the analysis. RNA was isolated with a RNeasy plus kit (*Qiagen,* Germany). Complementary deoxyribonucleic acid (cDNA) was synthetized with the Omniscript RT system (*Qiagen,* Germany), Oligo-dT primer (*Microsynth,* Switzerland) and RNasin Inhibitor (*Promega,* USA). Real-time RT-PCR was performed using SYBR-green (*Applied Biosystems,* USA) on a 7500 Fast Real-Time PCR system (*Applied Biosystems,* USA).

Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the standard (housekeeping) gene and filtered air as the control. The induction of cell death was determined by expression levels of (pro-)apoptotic genes caspase 7 (*CASP7*) and FAS receptor (*FAS*). Heme oxygenase 1 (*HMOX1*) and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*) genes were used to observe the onset of oxidative stress. For assessment of (pro-)inflammatory responses, expression of pro-interleukin-1 beta (*IL1B)* and interleukin-8 (*IL8*) genes were measured.

2.8. Data processing and statistical analysis

All data in the figures are presented as single values and means derived from gasoline exhaust or filtered air exposures (the initial exposures (n = 2) and repeated exposures (n = 2), over 4 days in total); each exposure was performed with two different sets of the multicellular lung model (n = 2; each exposed separately to volcanic ash), leading to 4 repetitions (n = 4).

All statistical analyses were performed using the software Origin (version 9.3, *OriginLab Corporation,* USA). Statistical significance was deduced through the use of a one-way analysis of variance (ANOVA), assuming a normal distribution of the datasets. Subsequent Tukey's post hoc tests were conducted to determine statistical significance between different exposures and the reference exposure (filtered air). The alpha value was set at 0.05.

3. Results

3.1. Volcanic ash characterisation

Particle size analysis of isolated respirable fractions showed that 98% by volume and 84% by volume of particles were sub-10 μm diameter, for ash from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV), respectively (Fig. 2a). The SHV sample consisted of 58% by volume particles with size <4 μm, while ChV contained less with 40% by volume.

The morphology of the particles from both volcanoes, as observed by SEM, was mostly blocky and angular with varying amounts of sub-micron particles adhering to the surfaces of larger particles (Fig. 2b). This is congruent with previous observations of respirable volcanic ash (*e.g.*, Damby et al., 2016, Lahde et al., 2013, Horwell et al., 2013, Hillman et al., 2012, Le Blond et al., 2010), but may not mirror the morphology of larger ash particles, which can differ according to (magmatic) composition.

Bulk oxide elemental data for samples are listed in Table 1 and indicate magmatic composition of the ash samples. The SHV ash was confirmed to be 'andesitic', with an intermediate composition regarding silicon dioxide (SiO_2) content (61.8 wt% SiO_2), while ChV is 'rhyolitic', being comparatively richer in SiO_2 (73.4 wt% SiO_2).

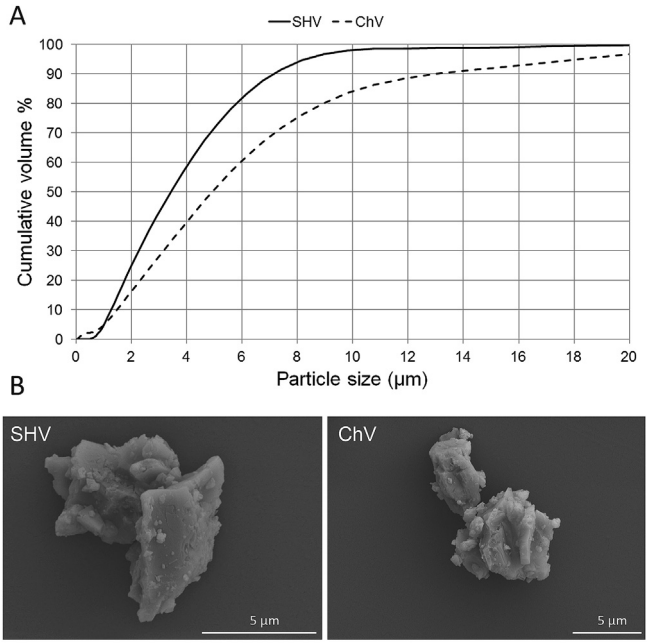

Fig. 2. Volcanic ash characterisation. A) Particle size distribution (PSD) of the isolated respirable fraction of volcanic ash samples determined by a Beckman Coulter LS 13 320 PSD analyser (*Coulter Corporation*, USA). Data are the mean of n = 3. **B)** Representative scanning electron microscopy images of volcanic ash samples from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV). Images were collected at 10.0 kV and WD 16 mm. Scale bars are 5 μm.

3.1.1. Volcanic ash nebulisation

The average cell-delivered doses of nebulized ash using the dry powder insufflator, as monitored by a QCM, were 0.54 ± 0.19 μg/cm^2 and 0.39 ± 0.09 μg/cm^2 for SHV and ChV ash, respectively (Fig. 3).

3.2. Exhaust characterisation

The composition of the gaseous fraction, comprising carbon monoxide (CO), total hydrocarbons (THC), non-methane hydrocarbons (NMHC), nitrogen oxides (NO_x) and carbon dioxide (CO_2), as well as the average count of produced particles are shown in Table 2.

3.3. Biological endpoints

For the assessed biological endpoints (cytotoxicity, oxidative stress and (pro-)inflammatory mediators, including measurements for both protein production and gene expression), no significant ($p > 0.05$) changes in cell cultures were observed at 24 h or 48 h time-points for any of the experimental exposures, *i.e.,* volcanic ash (VA), gasoline exhaust (GE) and co-exposures (GE + VA). The response of the untreated cells (*i.e.,* incubator control) was lower in comparison to the cells treated with filtered air, albeit not significant (SI Fig. 1). To account for the influence of the potentially stress-inducing airflow as well as for the different baseline levels in the various cultures, the comparison of the effects of cell treatments (VA, GE and GE + VA) was made with the filtered air (reference) exposure.

For all exposure scenarios (VA, GE and GE + VA) at 48 h, LSM imaging revealed a homogenous and confluent epithelial cell layer with no alteration in cell morphology compared to the filtered air (reference) exposure (Fig. 4a). LDH release by the cells following the exposures, for both time-points, showed limited elevation in

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Table 1
Bulk chemical compositions of the volcanic ash samples used in the study. Results are presented as component weight percent oxide and recalculated to include loss on ignition (LOI) in the final total.

Sample	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	SO_3	LOI	Total
SHV	61.8	0.5	17.0	6.6	0.2	2.4	6.3	3.7	0.9	0.1	0.1	0.6	100.1
ChV	73.4	0.2	13.9	1.6	0.1	0.4	1.5	4.2	2.9	0.1	0.0	1.1	99.4

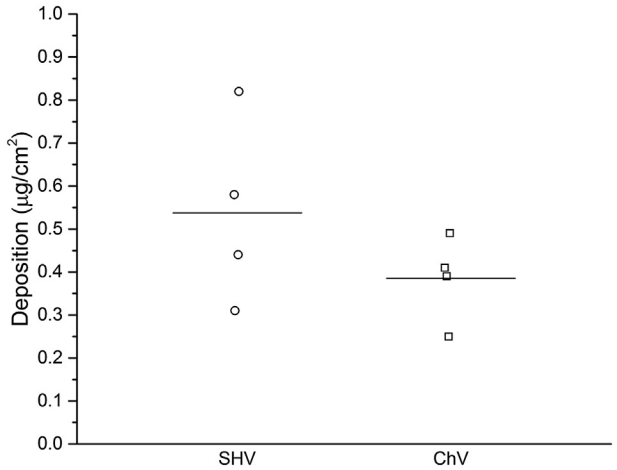

Fig. 3. Deposition of nebulized respirable volcanic ash. Average mass deposition ($\mu g/cm^2$) of respirable ash from Soufrière Hills volcano (SHV) and Chaitén volcano (ChV) quantified using a QCM, following their dry nebulisation over cell cultures using a dry powder insufflator (DP-4, *Penn Century*, USA). All data are presented as single values and mean (solid line), n = 4.

Table 2
Average exhaust composition for the flex-fuel GDI vehicle in the WLTC as measured during the experiments (n = 4). SD = standard deviation, CO = carbon monoxide, THC = total hydrocarbons, NMHC = non-methane hydrocarbons, NO_x = nitrogen oxides, CO_2 = carbon dioxide. *Note that the CO_2 concentration applied to the cell culture chamber was adjusted as necessary to 5% CO_2. PN = particle number. All data are shown 1:10 diluted (as applied to the cell cultures).

Exhaust component	Unit	Mean	SD
CO	ppm	27.71	2.98
THC	ppm	6.97	0.44
NMHC	ppm	4.82	0.46
NO_x	ppm	1.76	0.12
CO_2^*	%	0.98	0.01
PN	$\#/cm^3$	1.32E+05	1.65E+04

comparison to the filtered air (Fig. 4b). The positive LDH assay control Triton X-100 showed a significant ($p < 0.05$) increase in LDH content in culture medium, confirming that the biological model used was responsive for the measured endpoint.

For expression of pro-apoptotic genes *FAS* and *CASP7*, none of the exposures showed a statistically significant ($p > 0.05$) outcome relative to filtered air (Fig. 4c). *CASP7* expression was found to be slightly suppressed by VA, GE, and combined exposure to gasoline exhaust and SHV ash (GE + SHV), while only slightly increased in the combined exposure to gasoline exhaust and ChV ash (GE + ChV); these changes, however, are still within the observed biological variation.

As determined via release of specifically chosen (pro-)inflammatory mediators, none of the cell exposures induced a significant ($p > 0.05$) (pro-)inflammatory response (Fig. 5a). In fact, the concentrations of TNF-α and IL-1β in all measured samples were below the method detection limits (MDL; SI Fig. 1). LPS, which served as a positive assay control, significantly ($p < 0.05$) increased the release of IL-8 (Fig. 5a) as well as TNF-α and IL-1β (SI Fig. 2) compared to the filtered air and other cell treatments. Lack of a (pro-)inflammatory response was supported by the findings on a gene level, where the cell exposures did not induce any change in mRNA levels of measured markers, *IL8* and *IL1B*, relative to filtered air (Fig. 5b). In agreement with the protein measurements, none of the treatments induced a detectable upregulation of *TNFA* (data not shown). In the ChV ash-treated cultures, a slight, yet insignificant ($p > 0.05$), increase in expression of both *IL8* and *IL1B* was observed compared to the filtered air (reference) exposure.

Similarly, the expression of investigated genes related to oxidative stress, namely *HMOX1* and *NQO1*, showed no significant ($p > 0.05$) increase after exposure to VA, GE or GE + VA (Fig. 6). Combined exposure to gasoline exhaust and ChV ash (GE + ChV) did, however, result in a slight upregulation of *HMOX1* relative to filtered air, although not significant.

4. Discussion

The purpose of the study was to investigate the potential respiratory hazard of combined exposure to volcanic ash and anthropogenic pollution, through experiments designed to assess the impact to a multicellular lung model of exposure to both volcanic ash and complete gasoline exhaust.

We have found that exposure of cells to gasoline exhaust, alone, does not induce any significant effects on any of the biological endpoints measured. These results are in agreement with research performed by Bisig et al. (2016) using gasoline exhaust, alone, on a multicellular human lung model mimicking the bronchial compartment, under similar experimental conditions. Another study by Bisig et al. (2015), using the same experimental setup but a different car, found that gasoline exhaust induced oxidative stress; however, the particle number measured in the diluted exhaust was up to three orders of magnitude higher than that used by Bisig et al. (2016), and nearly twice as high as the average daily number of particles produced during exposures in the present study (Table 2). Similarly, the concentrations of volatile compounds in Bisig et al. (2015) exceed those measured in Bisig et al. (2016) and the current study. Bisig et al. (2015) found that filtration of the particulate fraction from the exhaust was not sufficient to eliminate the adverse effects *in vitro*, confirming the importance of the volatile compounds in GE-induced toxicity. The toxic effects of gasoline exhaust, particularly after particle filtration, have also been observed with *in vivo* animal studies (Reed et al., 2008; Lund et al., 2007). It was noted, though, that volatile compounds, alone, might react differently with the lung cells than when part of the complete exhaust, where these compounds can adsorb onto the particle surfaces (Steiner et al., 2014, 2016).

The biological sensitivity of the employed multicellular model has been validated in the past through use of positive particulate controls, *e.g.*, crystalline quartz (DQ12) for a (pro-)inflammatory response (Endes et al., 2014; Chortarea et al., 2015). The lack of adverse effects observed following gasoline exhaust exposure in the present study may be explained by important differences in experimental parameters, including the employed cell lines, the

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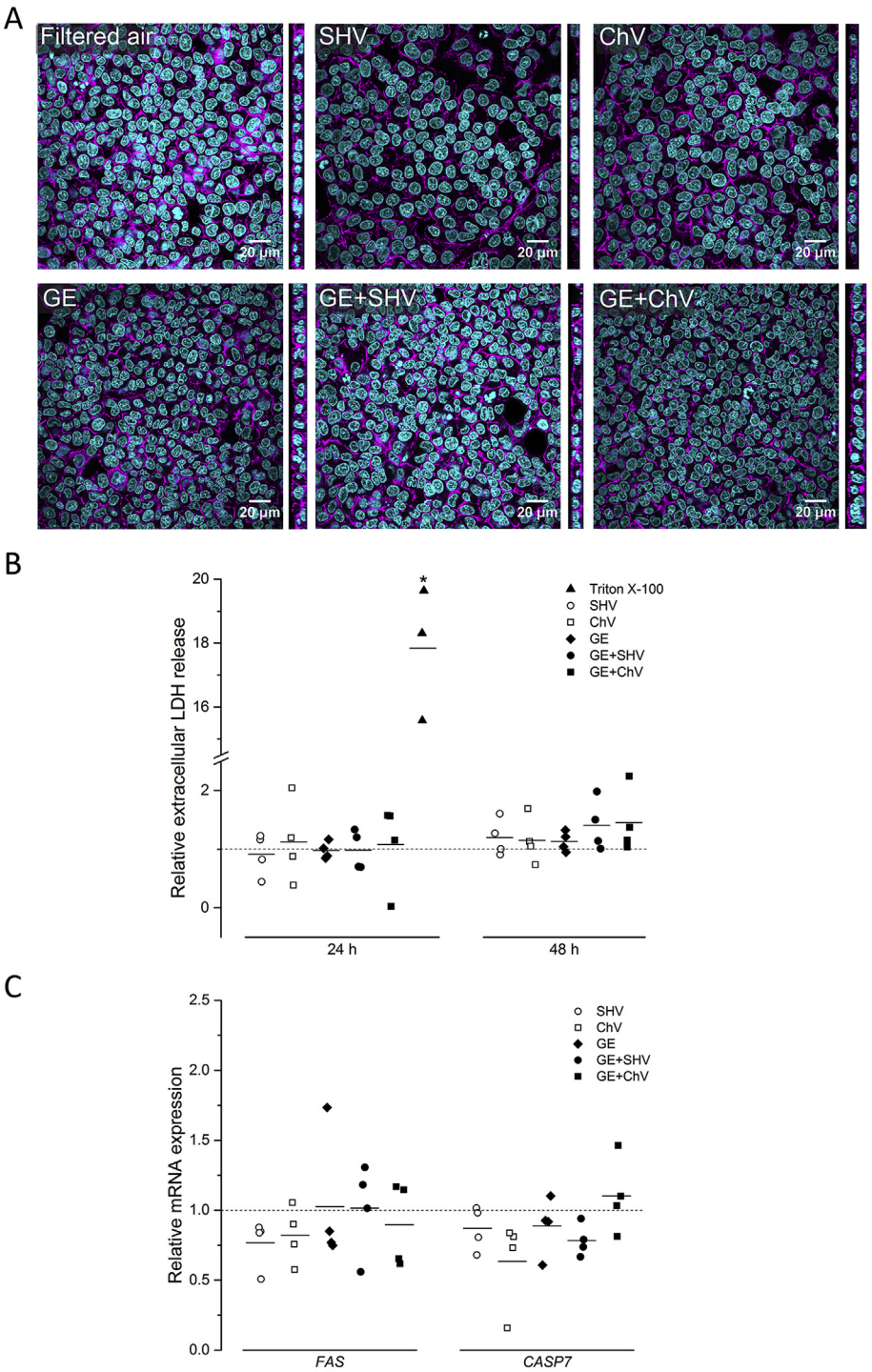

Fig. 4. Cell morphology and cell viability of the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. **A)** Representative confocal LSM images from XY and YZ projections for cultures exposed to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE + SHV), and combined exposure to gasoline exhaust and Chaitén ash (GE + ChV). Cells were stained with Phalloidin-Rhodamine (F-actin cytoskeleton, magenta) and DAPI (cell nuclei, cyan). Scale bars are 20 µm. **B)** Extracellular LDH levels in the culture medium after 24 h and 48 h normalized to filtered air (reference) exposure (dashed line). The positive assay control was 0.2% Triton X-100 in PBS. **C)** Amounts of mRNA of pro-apoptotic genes FAS receptor (*FAS*) and caspase-7 (*CASP7*), 48 h post-exposures, normalized to filtered air (reference) exposure (dashed line). All data are presented as single values and mean (solid line), n = 4; * denotes a significant difference ($p < 0.05$) between the positive control and the other samples tested. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

driving test cycle and vehicle tested and, hence, the lower particle numbers and volatile concentrations (and consequent lower doses) as compared to other studies, and exposure times. In addition, it is known that the response of cultured cells to exposure may vary, especially over time (Poland et al., 2014). The evidence from other studies such as Bisig et al. (2015) suggests that we should not conclude that gasoline exhaust is incapable of inciting a biological response.

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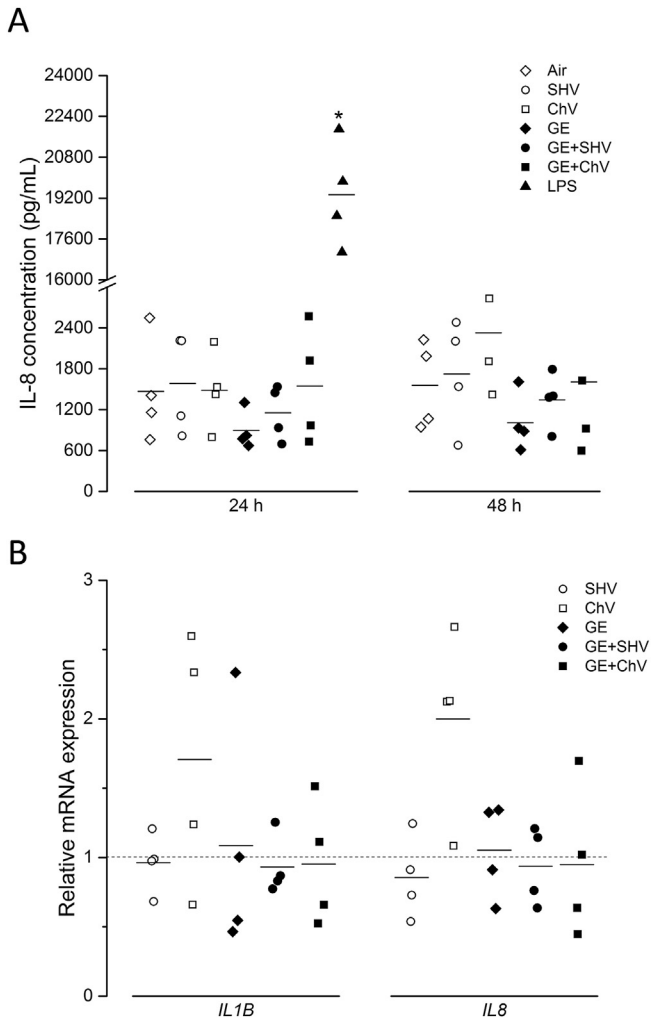

Fig. 5. Release of (pro-)inflammatory mediators in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. A) Interleukin-8 (IL-8) release in the culture medium after 24 h and 48 h following exposures to filtered air (reference exposure), (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE + SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE + ChV). The positive assay control was lipopolysaccharide (LPS; 1 μg/mL, 24 h). **B)** Amounts of mRNA of (pro-)inflammation-related genes encoding interleukin-1 beta (*IL1B*) and IL-8 (*IL8*), 48 h post-exposures, normalized to filtered air exposure (a dashed line). All data are shown as single values and mean (solid line), n = 4; * denotes significant difference ($p < 0.05$) between the positive control and the other samples tested.

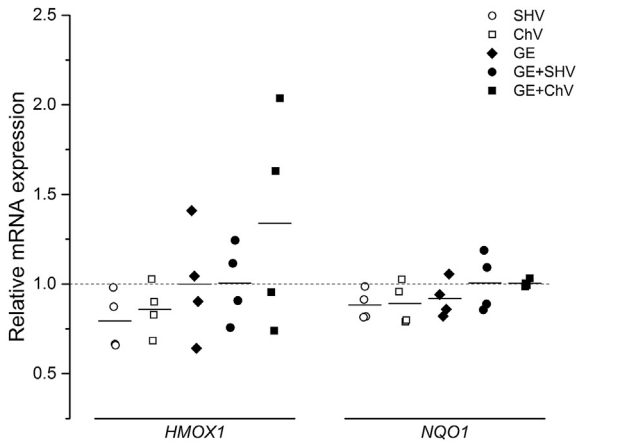

Fig. 6. Oxidative stress response in the multicellular lung model following combined exposure to gasoline exhaust and volcanic ash. Amounts of mRNA of oxidative stress responsive genes, heme oxygenase 1 (*HMOX1*) and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*), following exposures to (filtered air and) Soufrière Hills ash (SHV), (filtered air and) Chaitén ash (ChV), gasoline exhaust (GE), combined exposure to gasoline exhaust and Soufrière Hills ash (GE + SHV) and combined exposure to gasoline exhaust and Chaitén ash (GE + ChV), normalized to filtered air exposure (dashed line). Data are shown as single values and mean (solid line), n = 4.

The two ash types used in this study, erupted from different volcanoes of different magmatic compositions, also did not elicit a significant response in the biological endpoints measured from the multicellular model. Ash samples from these volcanoes have been previously investigated for their toxic potential and also showed limited biological responses (Damby et al., 2016, Horwell et al., 2013, Wilson et al., 2000, Cullen and Searl, 1998 and unpublished data for ChV). The SHV ash has also caused minimal response to the same multicellular model in our previous study (Tomašek et al., 2016). Hence, these data confirm the generally-observed lack of potential of ash to significantly affect healthy lung cell integrity or function including, in this case, initiation of an inflammatory response for the chosen time-points and endpoints.

Given the lack of significant response to gasoline exhaust, alone, and volcanic ash, alone, the finding that the co-exposures did not cause significant adverse effects in the multicellular model is perhaps not surprising, and could indicate that these combined exposures did not generate either an additive or synergistic response. Due to experimental design, the effect of direct ash-volatile interactions (*e.g.,* volatile adsorption) prior to co-culture exposures was unable to be tested here. The absence of alterations in cell morphology, cell viability and oxidative stress state for any combined exposure scenario are in line with our previous *in vitro* study, which showed limited cytotoxic and oxidative potential of SHV ash when exposed concomitantly with DEP (Tomašek et al., 2016). However, the low (pro-)inflammatory response following combined exposures is contrary to the previous findings, where co-exposures of SHV ash and DEP increased release of (pro-) inflammatory mediators TNF-α and IL-8, as well as significantly increased ($p < 0.05$) IL-1β (Tomašek et al., 2016). In Tomašek et al. (2016) it was hypothesized that the observed IL-8 production was driven by DEP, and that volcanic ash, alone, did not result in significant production of TNF-α, although it augmented TNF-α production in the co-exposures. Together with the results from Bisig et al. (2016), who report no upregulation of *TNFA* or *IL8* in response to gasoline exhaust exposure in a bronchial epithelium model, it appears that co-exposures to volcanic ash and gasoline exhaust do not induce inflammation via these pathways, at least at the doses tested here, and using the lung model composed of cells from healthy donors. However, the non-significant increase in *IL8* expression in response to ChV ash remains unexplained, especially since this sample has not been used in cytokine assays previously.

The similar biological responses to both ash samples in combined exposures with gasoline exhaust indicates that, within the parameters of this particular experimental setup, differences in sample composition (*e.g.,* iron content) and mineralogy (*e.g.*, crystalline silica content) did not affect the biological response to co-exposures. Whilst we chose two fairly different ash samples, volcanic ash is a heterogeneous dust, the physicochemical characteristics of which can vary considerably, even during a discrete eruption (Damby et al., 2017; Horwell et al., 2013), and samples from different eruptions have shown differences in toxicity when tested comparatively *in vitro*, previously (Damby et al., 2016; Horwell et al., 2013; Wilson et al., 2000). Hence, a different sample from an individual eruption or a different ash type might incite a different cellular response.

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The potential for diesel exhaust, and DEP in particular, to cause adverse respiratory effects is well known (see review by Steiner et al., 2016) while, on the contrary, the toxicity of exhaust from GDI vehicles is still relatively unknown (CCEM, 2016; Muñoz et al., 2016). Given this, we believe that there is a need to conduct further studies to clarify the hazard posed by combined exposures, particularly with a fuel that generates exhaust of likely greater toxicity (*e.g.,* diesel) (Bisig et al., 2016), which was not possible during the time-frame of the current experiments. Future studies that consider the very complex and variable components of ambient urban air would be prudent, as would additional endpoints, such as genotoxicity, that help derive a more comprehensive understanding of the potential hazard. Furthermore, the experimental approach in this study, although performed over a two-day period (as opposed to the commonly-used time-point of 24 h), still represents a short-term exposure scenario. Hence, potential chronic effects that such exposures could elucidate, over a prolonged period, have not been accounted for and need to be investigated.

5. Conclusion

This study provides the first insights into the biological effects caused by exposure to complete gasoline exhaust in the presence or absence of volcanic ash conducting a realistic *in vitro* hazard assessment. The findings show that combined, and individual, gasoline exhaust and volcanic ash exposure at the ALI has limited adverse biological impact to a multicellular lung model *in vitro*, considering the employed experimental conditions and biological endpoints measured (cytotoxicity, oxidative stress and (pro-)inflammatory response at the protein and gene levels).

More detailed investigation of the potential respiratory hazard following such combined exposures in future eruptive events is necessary, especially considering the complexity of the ambient urban air. Additional biological markers should be studied in further experiments *in vitro* and a complete diesel exhaust could also be used.

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Conflict of interest

The authors declare no conflict of interest. The authors are responsible for the content of the manuscript.

Consent for publication

All authors have read and approved the manuscript for publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.01.115.

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